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ABSTRACTS:
IN THIS ISSUE

PLASMA CHOLESTEROL CONCENTRATIONS IN SQUIRREL MONKEYS AS INFLUENCED BY DIET AND PHENOTYPE. H.B. LOFLAND, D.C. JONES, T.B. CLARKSON & R.W. ST. CLAIR. *J. Food Sci.* **40**, 2–7 (1975)–Monkeys making either a high (hyperresponders) or low (hyporesponders) plasma cholesterol response to dietary cholesterol were fed 0.75 mg cholesterol/cal of diet for 3 months. Dietary fat (40% of calories) was butter or safflower oil. Absorption of cholesterol and excretion of cholesterol and bile acids were measured. Hyporesponders absorbed significantly less dietary cholesterol than did hyperresponders, which may in part explain their lower plasma cholesterol response to cholesterol feeding. Bile acid excretion increased after the first month of cholesterol feeding; this may be one determinant of final plasma cholesterol concentration. There was no difference in per cent absorption of cholesterol between butter and safflower oil diets. However, those monkeys fed butter consumed more diet and absorbed more cholesterol than did those fed safflower oil. Plasma cholesterol concentrations also were higher for butter-fed monkeys.

NONNUTRITIVE FIBER AND LIPID METABOLISM. D. KRITCHEVSKY, S.A. TEPPER & J.A. STORY. *J. Food Sci.* **40**, 8–11 (1975)–Rats were fed semi-synthetic (SS) diets which contained cellulose as the nonnutritive fiber (NNF) and their serum and liver lipid levels were compared with those of chow-fed rats. Serum cholesterol and triglyceride levels were reduced by 35 and 62%, respectively. Liver cholesterol levels were 41% lower but triglycerides were unchanged. The rats were fed [^{14}C]-cholesterol and the chow group excreted 228% more labeled acidic steroid. Serum cholesterol levels were generally lower when alfalfa was substituted for cellulose in the SS diets. Serum plus liver cholesterol pools were also lower. After a dose of oral [^{14}C]-cholesterol the alfalfa fed animals excreted up to 82 and 108% more neutral and acidic steroids than did the cellulose fed rats. To ascertain whether alfalfa played a role in lipid absorption the in vitro binding of sodium taurocholate (NaTC) to alfalfa was investigated. Alfalfa bound 21 and 30% as much NaTC as did cholestyramine and colestipol, two preparations used specifically as oral hypocholesteremic agents. Cellulose bound 3% as much NaTC as did alfalfa. Several other types of NNF were also tested. Sugar cane pulp, bran and oat hulls bound significant amounts of NaTC. NNF appears to play an active role in cholesterol metabolism, either by interference with absorption, enhancement of oxidation or some other mechanism.

LIPIDS IN ATHEROSCLEROSIS. F.A. KUMMEROW. *J. Food Sci.* **40**, 12–17 (1975)–The presence of cholesterol and polyunsaturated fatty acids (PUFA) in culinary fats and oils (lipids) are considered important to the development of atherosclerosis because a decrease in the consumption of cholesterol containing animal fats and an increase in the consumption of PUFA has decreased serum cholesterol levels in human subjects. High serum cholesterol and high triglyceride (lipid) levels represent a risk factor in the development of atherosclerosis in the coronary arteries, the cause of 90% of all heart disease. However, the type of PUFA in the serum lipids and in the cell membranes in the arteries may be even more crucial to the development of atherosclerosis. In the process of preparing culinary fats and oils, the "essential" PUFA in natural fats are reduced in amount by hydrogenation and thus stabilized towards oxidative rancidity. In current practice, a major share of the PUFA are also converted to isomeric trans fatty acids. Rabbits, swine and human subjects fed fats which contained trans fatty acids had higher serum cholesterol levels and the aortas of these rabbits and swine had a higher cholesterol level than those fed natural fats. It therefore seems desirable to prepare culinary fats devoid of trans fatty acids.

EFFECTS OF DIETARY PROTEIN AND CARBOHYDRATE ON PLASMA CHOLESTEROL LEVELS IN RELATION TO ATHEROSCLEROSIS. K.K. CARROLL & R.M.G. HAMILTON. *J. Food Sci.* **40**, 18–23 (1975)–The hypercholesterolemia which develops in rabbits on cholesterol-free semisynthetic diets appears to be primarily dependent on the protein component of these diets, although the effect can be modified by dietary carbohydrate and dietary fat. There is also evidence that dietary protein and carbohydrate, as well as dietary fat, can influence the level of serum cholesterol in other animal species and in humans.

THERMAL EFFECTS ON THE LENGTH OF SARCOMERES IN MUSCLES HELD AT DIFFERENT TENSIONS. P.V.J. HEGARTY & C.E. ALLEN. *J. Food Sci.* **40**, 24–27 (1975)–The effect of heat on sarcomere length in muscle fibers held at different tensions was studied. Heat caused the greatest shortening in isolated fibers, less in dissected muscle and least in muscle attached to the skeleton. The greatest shortening due to heat occurred in the longest sarcomeres. When muscle strips of different thickness, each greater than 0.5 cm., were cut from the same turkey semitendinosus muscle and heated to the same degree of doneness, similar sarcomere lengths were obtained. Sarcomere length relationships in unheated turkey muscle which entered rigor at 20°C, 2°C and both pre- and post-rigor frozen/thawed samples persisted in the heated state. The investigation suggests that sarcomere length/tenderness relationships should be expressed for heated muscle plus reference to whether the muscle was attached or unattached to the bone when heated.

RELATIONSHIPS OF ELECTROPHORETIC PATTERNS AND SELECTED CHARACTERISTICS OF BOVINE SKELETAL MUSCLE AND INTERNAL TEMPERATURE. N.E. FOGG & D.L. HARRISON. *J. Food Sci.* **40**, 28–34 (1975)–Effects of internal temperature (25° and 45°C) on color of semitendinosus muscles and on electrophoretic patterns of heme pigments in the sarcoplasmic extracts were investigated. Measurements on raw and heated intact muscle were pH, total moisture, cooking losses, ether extract, color-difference and rate of heat penetration. Sarcoplasmic extracts were subjected to electrophoresis in polyacrylamide disc gels containing sodium dodecyl sulfate. Migration distances of selected components in extracts were measured from spectrophotometric scans (405 nm) of the gels. Heating increased pH, cooking losses and ether extract, and decreased moisture of intact muscle. Color-difference was altered slightly. Spectral and electrophoretic pattern changes of extracts could not be attributed to any single factor studied.

COMPOSITION AND PROTEIN EFFICIENCY RATIO OF PARTIALLY DEFATTED CHOPPED BEEF AND OF PARTIALLY DEFATTED BEEF FATTY TISSUE AND COMBINATIONS WITH SELECTED PROTEINS. M.L. HAPPICH, R.A. WHITMORE, S. FEAIRHELLER, M.M. TAYLOR, C.E. SWIFT, J. NAGHSKI, A.N. BOOTH & R.H. ALSMEYER. *J. Food Sci.* **40**, 35–39 (1975)–Proximate and amino acid composition and the protein efficiency ratio (PER) were determined for partially defatted (PD) chopped beef and PD beef fatty tissue, commercial products from low temperature rendering of beef tissues, to evaluate their protein nutritive quality. Lean beef was included as a reference protein. PER values for PD chopped beef ranged from 1.61–2.58 and for PD beef fatty tissue, 1.13–1.70. Results indicate that the use of a PD cured cooked chopped beef product and of one PD chopped beef product, having PER values equal to or not significantly different from that of

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casein, are nutritionally acceptable as protein sources. PER's of the products studied were directly correlated with the essential amino acid composition. Data indicate that tryptophan is the most limiting essential amino acid in the PD products and that small amounts had a strikingly beneficial effect on the PER value. PER's for mixtures of two PD beef fatty tissue products individually with lean beef, a whey or a soy protein concentrate were significantly higher than those of the beef fatty tissues individually, ranging from 1.99–2.72. The products would be acceptable as food ingredients when blended with protein foods in which amino acid compositions are mutually supplementary.

PHYSICO-CHEMICAL CHARACTERIZATION OF NORMAL AND PSE PORCINE MUSCLE MYOGLOBINS. M. BEMBERS & L.D. SATTERLEE. *J. Food Sci.* **40**, 40–43 (1975)–The instability of myoglobin from pale, soft and exudative (PSE) muscle was noted in this study by its: (1) varying isoelectric points; (2) rapid rate of autoxidation, (3) heat instability of both met and oxy forms; (4) acid instability; and (5) lower α-helical content. The question as to whether this abnormal myoglobin is a result of genetic change or a result of the rapid pH drop that occurs in PSE muscle upon death was left unanswered.

Enterobacteriaceae AT VARIOUS STAGES OF POULTRY CHILLING. N.A. COX, A.J. MERCURI, B.J. JUVEN & J.E. THOMSON. *J. Food Sci.* **40**, 44–46 (1975)–Broiler carcasses at three locations (before chilling, after slush-ice chilling and after slush-ice then air-blast chilling) during chilling plus chiller water were sampled for types and numbers of *Enterobacteriaceae* and total plate count. Total plate count of the water in the chiller was log 3.26 per ml. Carcass skin total count was log 3.17 per cm^2 before chill then declined to 2.64 after chilling. *Escherichia* predominated among *Enterobacteriaceae* at all locations. Other genera less frequently found were *Enterobacter, Klebsiella* and *Providencia.* During 10-day storage at 4°C, *Enterobacteriaceae* count per cm^2 increased from approximately log 1 to almost log 4; *Escherichia* declined from 85% of *Enterobacteriaceae* before storage to 14% after storage, while *Enterobacter* increased from 6 to 83%.

EFFECT OF GAMMA RADIATION ON CHICKEN LIVER CATHEPTIC ACTIVITY AND RELEASE OF LYSOSOMAL CATHEPSIN D. M. ALI & J.F. RICHARDS. *J. Food Sci.* **40**, 47–49 (1975)–Chicken liver slices were subjected to 0.05–1.00 Mrad doses of gamma radiation. Total and free catheptic activities were determined immediately after irradiation. Total catheptic activity was higher and free catheptic activity was lower in irradiated samples than in nonirradiated controls. Isolated lysosomal suspensions were irradiated at doses 0.1–1.00 Mrad. Total catheptic activity was significantly decreased by irradiation. Radiation resulted in a decrease in absorbance (540 nm) of lysosomal suspensions incubated at 37°C which was paralleled by a pronounced increase in free catheptic activity. Rate of release of catheptic enzymes from lysosomes was considerably slower when incubated at 4°C compared to 37°C.

EFFECT OF VACUUM PACKAGING ON MICROORGANISMS ON CUT-UP CHICKENS AND IN CHICKEN PRODUCTS. A.S. ARAFA & T.C. CHEN. *J. Food Sci.* **40**, 50–52 (1975)–Packaging cut-up fresh broilers in 1.5 mil polyethylene pouches with 25 psi guage vacuum did not enhance the shelf life of the product. Physiological and biological characteristics of the isolates indicated that 95.9% of the microorganisms found in vacuum packaged parts after spoilage were members of the genus *Enterobacter,* while only 4.1% were found to be pseudomonads. In nonsealed control samples. however, members of the genus *Pseudomonas* were the dominant bacteria responsible for spoilage and only 4.2% were found to be the *Enterobacter* species. Using pure cultures, three selected members of the family *Enterobacteriaceae (Aerobacter aerogenes, Escherichia coli* and *Proteus mirabilis)* were found to survive and grow on the vacuum packaged poultry meat at 2–4°C.

CORRELATION BETWEEN THE FRESHNESS OF ICED SALMONIDS AND LACTIC ACID FORMATION IN MUSCLE EXTRACTS. E. BILINSKI & R.E.E. JONAS. *J. Food Sci.* **40**, 53–57 (1975)–The relationship between the postmortem age of fish and the formation of lactic acid by soluble muscle enzymes was investigated in rainbow trout (*Salmo gairdneri*) and sockeye salmon (*Oncorhynchus nerka*) held in melting ice for 0–14 days. The test consisted in measuring the conversion of fructose-1,6-diphosphate to lactic acid in muscle extracts, in presence and absence of added nucleotides. In absence of added nucleotides, lactic acid formation showed a pronounced decrease within the first week of storage. When an assay containing ATP was used, the activity was maintained for several more days, whereas in presence of added NAD^+ or NADH it remained at comparatively high levels during 14 days of ice storage. Significant differences ($P \leq 0.02$) were evident between freshly killed fish and fish held in ice for 3, 7 and 14 days.

EFFECT OF MOISTURE, CARBOHYDRATES AND ATMOSPHERE ON THE FUNCTIONAL STABILITY OF FISH PROTEIN ISOLATES. B.J. KOURY & J. SPINELLI. *J. Food Sci.* **40**, 58–61 (1975)–Studies were conducted on the factors affecting the functional storage stability of fish protein isolates that were prepared by complexing partially hydrolyzed myofibrillar fish proteins with sodium hexametaphosphate. The stability of the isolates as reflected by a change in emulsifying capacity was related to their moisture content and reached a maximum at about 2.5% moisture. The stability of the isolates was enhanced by co-drying with nonreducing sugars such as sucrose and lactose. Reducing sugars offered no protection. Moisture absorption and desorption curves of the isolates showed that carbohydrates had the most significant effect on the moisture isotherm between ERH 12.0 and 57. Storage in nitrogen and vacuum atmospheres did not affect the stability of the isolates.

PREPARATION AND EVALUATION OF SOY-FORTIFIED GLUTEN-FREE BREAD. G.S. RANHOTRA, R.J. LOEWE & L.V. PUYAT. *J. Food Sci.* **40**, 62–64 (1975)–Studies were undertaken to develop a formula for the production of gluten-free bread for individuals suffering from gluten-intolerance. The addition of 20–40% soy protein isolate to unmodified wheat starch not only raised the protein content of the resultant breads substantially but improved the physical characteristics as well. These breads had good volume and appearance, exhibited excellent internal characteristics, compared very favorably with wheat bread in taste and flavor and were inexpensive to produce. All starch breads were appreciably higher in moisture content than the wheat bread. Protein quality as assessed by rat-feeding studies showed that the protein efficiency ratio of soy-fortified starch-breads was appreciably higher than that of the wheat bread.

EVALUATION OF PROTEIN QUALITY OF IRRADIATED FOODS USING Tetrahymena pyriformis W. AND RAT ASSAY. H. SRINIVAS, U.K. VAKIL & A. SREENIVASAN. *J. Food Sci.* **40**, 65–69 (1975)–Protein quality of irradiated foods was evaluated with *Tetrahymena pyriformis* W. as the test organism. Growth response of the organism depended upon protein quality and was not affected when grown for four successive transfers in media containing irradiated foods. The poor growth on wheat proteins could be improved by supplementation with limiting amino acids, namely, lysine, threonine and methionine either singly or in combination. Protein quality was also assessed by the conventional rat assay procedure and results with *Tetrahymena* were in agreement with these values.

SELECTED FUNCTIONAL PROPERTIES OF SUNFLOWER MEAL (Helianthus annuus). V.L. HUFFMAN, C.K. LEE & E.E. BURNS. *J. Food Sci.* **40**, 70–74 (1975)–Sunflower meal exhibits excellent functionality for possible use in specialized food. The most promising properties are emulsion capacity, water adsorption, water retention and aeration properties. With low mixing speeds and rapid rates of oil addition, optimum emulsion capacity occurs at pH 7. Water adsorption capacity increases as the native protein concentration increases among varieties. Optimum foam volume and stability are produced at pH 9 with a meal concentration of 8%, and a whipping time of 12 min at 15°C. At this pH, foams are a definite green color due to the oxidation of chlorogenic acid. However, the combination of sucrose and potassium bitartrate added to the foam produces a bright white foam with excellent volume and stability.

PROTEIN QUALITY AND SUPPLEMENTARY VALUE OF COTTONSEED FLOUR. M.L. HARDEN & S.P. YANG. *J. Food Sci.* **40**, 75–77 (1975)–Amino acid analyses and rat growth studies were used to determine the quality of proteins in glanded, glandless and liquid cyclone processed (LCP) cottonseed flours using casein and soybean oil meal as references. The cottonseed proteins contained approximately the same amount of methionine and threonine but slightly less lysine as compared to soybean and casein. The greatest deviation in net protein utilization (NPU) for all diets occurred at the 10% protein level, while NPU values varied slightly at 20% protein levels. Young rats fed the glanded cottonseed flour died within 5 days. When LCP and glandless cottonseed flours were substituted for 18.8% of wheat flour in a yeast bread, the protein content increased from 10.48% to 19.06% and 21.13%, respectively. Amino acid data and NPU value showed that LCP and glandless cottonseed flours made significant improvement to the nutritive value of a wheat flour yeast bread.

AN ENZYMATIC-CHEMICAL METHOD FOR EXTRACTION OF COTTONSEED PROTEIN. E.A. CHILDS. *J. Food Sci.* **40**, 78–80 (1975)–Experiments were undertaken to improve the extraction of protein from heat-treated cottonseed meal by treating the meal with proteolytic enzymes. A two-stage chemical technique (water and 0.075% NaOH) extracted approximately 15% of the cottonseed meal protein. Papain treatment did not markedly increase the amount of protein extracted but trypsin treatment increased protein extraction fivefold. The increased efficiency of trypsin resulted from the increased amounts of protein extracted in the NaOH fraction. Greater than 50% of cottonseed protein could be extracted from meal held at 204°C for 30 min.

FUNCTIONAL PROPERTIES OF THE GREAT NORTHERN BEAN (Phaseolus vulgaris) PROTEIN ISOLATE. L.D. SATTERLEE, M. BEMBERS & J.G. KENDRICK. *J. Food Sci.* **40**, 81–84 (1975)–Extraction of Great Northern beans (*Phaseolus vulgaris*) with a NaCl solution yields a high protein powder (Bean Protein Concentrate–BPC). The BPC is composed of 65% globulins and 35% albumins. The emulsion capacity of the albumins is good, as is the foam stability. The globulins possessed the best foaming ability, although being poorer than the albumins in foam stability. When the BPC was added to white bread at high levels, a major decrease in loaf volume occurred. Addition to a soft wheat flour cookie formulation improved the spread of the cookies during baking.

BIOCHEMICAL PROPERTIES OF ALFALFA PROTEIN CONCENTRATE. B.L. FREE & L.D. SATTERLEE. *J. Food Sci.* **40**, 85–89 (1975)–Several biochemical parameters of alfalfa protein concentrate (APC) were investigated in this study. Included was the characterization of proteins in APC. Polyacrylamide gel electrophoresis demonstrated the presence of several proteins in alfalfa juice with the majority of those proteins shown to be water soluble in nature. Five isoelectric focusing components were observed, four of which were in the acidic pH range. Chlorogenic acid, isolated from the alfalfa juice, was identified as the component of the alfalfa juice responsible for a color problem observed with APC. At an acidic pH, APC is a tan color, but above pH 6.0, the concentrate turns a bright yellow-green. Filtration of the alfalfa juice with activated charcoal and the addition of sodium sulfite to the alfalfa prior to pressing the juice were suggested as a means of alleviating the color problem. Heat-acid precipitation of the proteins in alfalfa juice is shown to be more efficient than heat precipitation alone.

TRITICALE, SOY-TVP, AND MILLET BASED DIETS AS PROTEIN RESOURCES FOR HUMAN ADULTS. C. KIES, H.M. FOX & L. NELSON. *J. Food Sci.* **40**, 90–93 (1975)–The objectives of the studies were as follows: (1) To define the relative protein value of the three test sources of protein (triticale, millet and soy-TVP®) for human adults when fed as single sources of dietary protein; (2) To determine the effect on protein value of substituting triticale for millet at two levels; (3) To determine whether the change in apparent protein value as a result of changing the ratio of millet to triticale flour was due to a change in lysine content or to changes in nonspecific nitrogen content; and (4) To compare the effect of addition of triticale or millet flours to a soy-TVP based diet on protein value. The nitrogen balance technique was used as the primary method of evaluation. Young adults, six to seven individuals for each of four 18-day studies, were subjects. Results indicated a distinctly better protein value of soy-TVP and the triticale flour in comparison to the millet flour. An improvement in protein value was achieved by mixing triticale with millet flour. This effect was found to be primarily the result in increasing the total protein content of the product, although the increase in lysine content also had a positive effect. Triticale flour was found to have a greater supplementary effect on the soy-TVP diet than did the millet flour protein although millet flour did not have a significantly adverse effect under these circumstances.

INFLUENCE OF TEMPERATURE ON KINETIC PROPERTIES OF PHOSPHORYLASE FROM TWO VARIETIES OF POTATO TUBER. G.W. CHISM, N.F. HAARD & P. WEISS. *J. Food Sci.* **40**, 94–96 (1975)–The effect of temperature on the kinetics of starch degradation catalyzed by phosphorylase was studied using extracts from two varieties of potato (*Solanum tuberosum*) tubers: "Kennebec" which tends to accumulate reducing sugars when stored at low temperatures and "Monona" which does not tend to accumulate reducing sugars when stored at low temperatures. The Km for phosphate increased from 6.6 to 10 mM when the assay temperature was reduced from 25°C to 15°C or 5°C for extracts from cold-stored Kennebec tubers, but was unchanged in extracts from cold-stored Monona tubers. The Arrhenius plot for extracts from cold-stored Kennebec tubers had a break near 12°C showing an increase in the activation energy at low temperatures. The Monona extracts gave characteristic straight line Arrhenius plots. The presence of 1 mM C-AMP did not affect the kinetics of starch degradation by extracts from cold-stored Kennebec tubers.

APPARENT VISCOSITY AS A MEASURE OF MOIST MOUTHFEEL OF SWEET POTATOES. V.N.M. RAO, D.D. HAMANN & E.G. HUMPHRIES. *J. Food Sci.* **40**, 97–100 (1975)–Using a rotational viscometer, apparent viscosities of sweet potato purees from baked roots of eight cultivars were determined at 10 angular velocities on five dates during the fall of 1973. Baked roots of the cultivars were also evaluated for moist or dry mouthfeel by a taste panel on each of the test dates. Apparent viscosities and taste panel ratings were highly correlated indicating that viscosity is a measure of moist or dry mouthfeel of sweet potatoes. Any of the 10 angular velocities was suitable for comparison as

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long as the same velocity was used for all the cultivars. All of the cultivars tended to become more moist in mouthfeel with curing and increased storage time.

DIGESTIBILITY AND SAFETY OF LIMED HIDE COLLAGEN IN RAT FEEDING EXPERIMENTS. R. WHITMORE, A. BOOTH, J. NAGHSKI & C. SWIFT. *J. Food Sci.* **40,** 101–104 (1975)–Knowledge of the digestibility and safety of collagen was considered a prerequisite to the development of uses for it as a binder or texturizer for human food. Rat feeding tests showed that previously limed hide collagen is 90% digestible. Caloric availability was found to be 86% that of casein. Collagen was not toxic when fed at levels of 10 or 20% of the diet. Some evidence indicated that collagen fed at 20% of diet had a potential for producing diarrhea, elevated hemoglobin or sensitivity to ether anesthesia.

EVALUATION OF LACTOSE AS A TRANSFER CARRIER FOR VOLATILE FLAVOR CONSTITUENTS. K. YABUMOTO, W.G. JENNINGS & R.M. PANGBORN. *J. Food Sci.* **40,** 105–108 (1975)–The efficiency of lactose as a transfer agent for banana flavor volatiles originally trapped from a gas stream on Porapak Q was investigated. Although the levels of volatile materials desorbed from the Porapak traps exceeded the adsorptive capacity of the lactose, low transfer temperatures and sealed storage produced a lactose carrier heavily impregnated with the flavor volatiles. On exposure to the atmosphere at room temperature, the impregnated lactose lost volatiles, initially at a fast rate, and subsequently at a sustained rate suggesting a first-order process. Sensory analyses indicated that an aroma termed "artificial banana" was highly correlated with integrated peak areas of the gas chromatograms. Overall odor intensity correlated highly with the butyrate esters, but not with the acetate esters.

IMMOBILIZATION OF PAPAIN ON COLLAGEN AND THE USE OF COLLAGEN-PAPAIN MEMBRANES IN BEER CHILL-PROOFING. K. VENKATASUBRAMANIAN, R. SAINI & W.R. VIETH. *J. Food Sci.* **40,** 109–113 (1975)–Papain (E.C. Number 3.4.4.10) was immobilized on a proteinaceous carrier, collagen, by two different methods: covalent binding and complexation. The conjugated papain-collagen membranes were used to effect the hydrolysis of both a low molecular weight substrate, benzoyl-L-arginine ethyl ester (BAEE), and a high molecular weight substrate, gelatin. For both classes of hydrolysis reaction, kinetic data obeyed pseudo-first order kinetics over the range of substrate concentrations examined in this study. The expressed catalytic activity of covalently bound papain-collagen membrane was 4.5-fold lower than that of a membrane prepared by the complexation process. The stability and reusability of collagen-papain membranes tested with BAEE and gelatin substrates in a spiral-wound biocatalytic modular reactor configuration were found to be very good. The use of a papain-collagen biocatalytic reactor in chill-proofing beer was investigated. One month and 3 months chill-haze tests, conducted with beer processed through the reactor in continuous operation, indicated satisfactory levels of chill-proofing. There was no alteration in the taste, color and foam stability of the processed beer. After 5 months of extensive continuous and intermittent usage in chill-proofing beer, the biocatalytic module retained 56% of its original activity.

UTILIZATION OF OLIGOSACCHARIDES BY LACTIC ACID BACTERIA DURING FERMENTATION OF SOY MILK. B.K. MITAL & K.H. STEINKRAUS. *J. Food Sci.* **40,** 114–118 (1975)–Utilization of oligosaccharides during fermentation of soy milk with lactic acid bacteria was studied. Trimethylsilyl derivatives of sugars were prepared and analyzed by gas-liquid chromatography. Fermentation of soy milk with *Lactobacillus cellobiosis* resulted in complete utilization of sucrose and raffinose within 20 hr. Stachyose concentration decreased 35% during this period and remained unchanged thereafter. From 6 hr onwards an unknown compound identified as mannitol appeared which was likely formed by the reduction of fructose released during sucrose hydrolysis. *Lactobacillus plantarum* completely utilized sucrose within 30 hr whereas raffinose content decreased slightly during early stages of fermentation and remained constant thereafter. Only 60% of the stachyose was utilized. Fermentation of soy milk with *Lactobacillus fermenti* showed complete utilization of raffinose and stachyose within 12 and 25 hr, respectively. Sucrose was not fermented. Fermentation of soy milk with a mixed culture of *Streptococcus thermophilus* and *L. fermenti* resulted in complete utilization of raffinose and stachyose while 0.08% of sucrose remained unfermented.

Staphylococcus aureus GROWTH AND SURVIVAL IN MACARONI DOUGH AND THE PERSISTENCE OF ENTEROTOXINS IN THE DRIED PRODUCTS. W.H. LEE, C.L. STAPLES & J.C. OLSON JR. *J. Food Sci.* **40,** 119–120 (1975)–Enterotoxigenic strains of *Staphylococcus aureus,* tap water and semolina were blended together to study the potential for growth and enterotoxin formation in pasta dough. *S. aureus* grew well in a lump of wet dough or noodle-shaped product with or without 5.5% egg yolk. Enterotoxins A and C were detected repeatedly in the dough after 14–30 hr of incubation at 25° and 35°C. With an initial population of 10^9/g, *S. aureus* was nonviable after 6 months of storage at 25°C. In contrast, enterotoxins A and C and *Salmonella typhimurium* cells were stable in the dried products and were recovered after 1 yr of storage.

DEVELOPMENT OF A CHEMICAL TEST FOR SHRIMP QUALITY. B.F. COBB III & C. VANDERZANT. *J. Food Sci.* **40,** 121–124 (1975)–The total volatile nitrogen/amino nitrogen (TVN/AA-N) ratio had a significant negative correlation ($r = -0.97$, $P < 0.01$) with the potential shelf life of brown shrimp (*Penaeus aztecus*). The TVN/AA-N ratio had a significant correlation ($r = 0.81$, $P < 0.01$) with total plate counts (TPC) of acceptable quality brown shrimp and white shrimp (*Penaeus setiferus*). The correlation between the TVN/AA-N ratio and TPC may be indirect. The TVN/AA-N ratio increases at the same rate as the log of the TPC until bacterial spoilage begins, and then increases at a very rapid rate. Until bacterial decomposition is measurable, the TVN/AA-N ratio increases because of enzymic ammonia production. Extraneous ammonia, produced from glutamine and asparagine during analysis, can produce high TVN values. The use of saturated Na_3PO_4 as releasing agent in the Conway microdiffusion procedure prevents interference of glutamine and asparagine in TVN analysis.

A TOTAL REDUCING SUBSTANCE TEST FOR ASCERTAINING OYSTER QUALITY. J.A. LIUZZO, S.C. LAGARDE, R.M. GRODNER & A.F. NOVAK. *J. Food Sci.* **40,** 125–128 (1975)–A test for oyster

quality has been developed which measures the group of compounds designated as total reducing substances (TRS). The analysis utilizes the quantitative determination of both volatile and nonvolatile substances in oysters which are capable of reducing an alkaline solution of potassium permanganate. The applicability of the TRS test was determined by comparison with existing indices of quality, namely pH, trimethylamine-nitrogen, total volatile bases, indole, microbiological counts and organoleptic scores. Statistical analyses and correlations of the comparisons indicate that the TRS test provides a useful, reliable means of ascertaining the quality of ice-stored oysters.

EFFECTS OF TEMPERATURE-TIME COMBINATIONS ON DONENESS AND YIELDS OF WATER-COOKED BROILER THIGHS. C.E. LYON, B.G. LYON, A.A. KLOSE & J.P. HUDSPETH. *J. Food Sci.* **40,** 129–132 (1975)–In commercial poultry cooking, doneness and yield are of major concern. This study was designed to investigate temperature-time combinations as they affect doneness and yield of broiler thighs. End-point temperatures ranged from 73.8–87.8°C and holding time at the end-point temperatures from 0–30 min. Water at 90.5°C was used to heat pieces to desired end-point temperatures; they were then transferred to a water medium maintained at that end-point temperature for one of the seven selected holding times. Doneness data were collected from two sources: subjective data from two trained sight panels and objective data from the Hunter Color and Color Difference Meter. Weights of thigh pieces were recorded prior to cooking and after ice-slush cooling for yield data. Temperatures and times of cooking affected the Hunter a_L values (measure of redness) of the thigh pieces interdependently. There was a general trend for Hunter a_L values and cooked yields to decrease as end-point temperatures and holding times increased. Hunter a_L values for pieces at 87.8°C were the lowest in the study, and holding time at this end-point temperature had only slight doneness value (reduction in a_L score) and was detrimental to yield. Cooked yields ranged from 90 to 78% and while there was a general trend of reduced yields with increasing end-point temperatures and holding times this decrease was not proportionate to cooking time as measured in this study. The color data suggest end-point temperatures ranging from 82.2–87.8°C with decreased holding time as the end-point temperature increases to water cook broiler thighs.

SUBJECTIVE AND OBJECTIVE METHODS FOR ESTIMATING DONENESS IN WATER-COOKED BROILER THIGHS. B.G. LYON, C.E. LYON & A.A. KLOSE. *J. Food Sci.* **40,** 133–136 (1975)–Subjective and objective methods were investigated to find a suitable quality control method to monitor doneness in commercially cooked broiler thighs. Two sensory panels underwent extensive training in a modified descriptive analysis technique. Of the several visible attributes of doneness, both panels selected color as the primary indicator for doneness evaluations, but differed in the selection of the area (meat tissue or artery) to be given primary consideration. Responses were made on or between points on 60-unit linear scales subdivided by descriptive terms or symbols. Hunter Color Values of the tissues evaluated by the panelists were also determined. Each panel group was highly trained, but Panel 2 assigned greater changes in doneness than Panel 1 for the same changes in Hunter a_L values, and also required lower a_L values for doneness. Doneness scores from both panels were highly correlated with Hunter a_L (redness) values. Measurement of color with Hunter a_L values could be an objective method to monitor doneness.

INHIBITION OF Aspergillus niger IN AN INTERMEDIATE MOISTURE FOOD SYSTEM. K.M. ACOTT & T.P. LABUZA. *J. Food Sci.* **40,** 137–139 (1975)–The interaction of pH with several food additives was studied in an intermediate moisture food (IMF) to determine their efficacy against *A. niger*. An IMF chicken-based product was prepared to a water activity (a_w) of 0.85. To one system, citric acid was added to change the pH from 5.7 to 4.2. These systems were then blended with the antimycotics tested including potassium sorbate, calcium propionate, methyl and propyl paraben, 1,3-butanediol, glycerol, mannitol, sorbitol, propylene glycol, benzoic acid and pimaricin at 2–3 levels. To each system spores of *A. niger* were added to give a count of 10^4/g. The systems were stored in desiccators at a_w 0.85 and 23°C and time for mycelia to appear was measured over a 9-month period. At least 10 separate samples were used. The results showed that most of the growth inhibitors were as effective as potassium sorbate below the accepted FDA approved levels of addition. For example, calcium propionate at pH 5.7 and 0.3% w/w was as effective as the sorbate. Glycerol at 1.0% was effective at both pH levels. This study showed that many approved food additives not used in IMF systems are as effective as potassium sorbate in preventing the growth of a mold.

COMPOSITIONAL AND METABOLIC GROWTH EFFECTS IN THE BOVINE. Muscle, Subcutaneous and Serum Fat Classes. A.L. HECKER, D.A. CRAMER, D.K. BEEDE & R.W. HAMILTON. *J. Food Sci.* **40,** 140–143 (1975)–Muscle, subcutaneous fat and blood serum were collected periodically from 27 calves from birth to slaughter to study effects of growth on lipid classes. Extracted lipids were separated into classes with thin layer chromatography. Muscle fat classes underwent several changes. Phospholipids, free fatty acids and cholesterol decreased and triglycerides increased. The majority of the changes occurred by 8–10 months of age. The effect of growth on subcutaneous fat classes was negligible except with phospholipids and free fatty acids, which decreased. Serum lipid classes displayed reasonably constant concentrations during growth. Breed and sex effects on muscle, subcutaneous and serum fat classes were of a minor and inconsistent nature.

COMPOSITIONAL AND METABOLIC GROWTH EFFECTS IN THE BOVINE. Muscle, Subcutaneous and Serum Total Fatty Acids. A.L. HECKER, D.A. CRAMER & D.F. HOUGHAM. *J. Food Sci.* **40,** 144–149 (1975)–The composition of fatty acid profiles were determined on muscle, subcutaneous and serum lipids from 28 days of age to slaughter on 27 cattle. Significant increases in C14:0, C14:1, C16:1 and C18:1 and decreases in C18:0 and C18:2 occurred in muscle lipids with increase in age. In subcutaneous fats, unsaturated fatty acids increased and except for C16:0, saturated fatty acids decreased. Serum fatty acids were highly variable except for a significant decrease in C14:0 and C16:0. Serum lipid was much more saturated than muscle or subcutaneous fats. Breed and sex effects were small and inconsistent. Periods of greatest change were during the first 12 months of age.

CHANGES IN TENDERNESS AND COLLAGEN OF BEEF SEMITENDINOSUS MUSCLE HEATED AT TWO RATES. M.P. PENFIELD & B.H. MEYER. *J. Food Sci.* **40,** 150–154 (1975)–Changes in beef semitendinosus cores and intramuscular connective tissue (CT) heated in a water bath programmed to simulate oven roasting of 2 kg top rounds at 93 and 149°C to end points of 40, 50, 60 and 70°C were evaluated. Slow heating and higher end points produced more tender cores and resulted in greater solubilization of hydroxyproline containing materials. Proteolytic activity was present in cores heated to all end points. Prolonged proteolytic activity in slowly heated meat may promote increased tenderness. Solubilization of hydroxyproline from CT heated in buffer was greater at the slow rate and at higher end points. Solubility of CT in guanidine hydrochloride decreased with heating and to a greater extent at the slow rate of heating. It appears that solubilization of hydroxyproline containing materials from CT is not the only factor affecting the increased tenderness of slowly heated meat.

FUNCTIONAL PROPERTIES OF PROTEINS ISOLATED FROM BOVINE BLOOD BY A CONTINUOUS PILOT PROCESS. P.T. TYBOR, C.W. DILL & W.A. LANDMANN. *J. Food Sci.* **40,** 155–159 (1975)–Slaughter animal blood yields two distinct protein isolates, the plasma and globin. Protein accounts for better than 90% of each isolate weight. The proteinaceous material is a source of all the essential amino acids but the globin is limited by low levels of isoleucine and methionine. Isolates contained low levels of aerobic bacteria and were free of salmonella,

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shigella and staphylococci. The solubility of the plasma isolate proteins was dependent upon spray drying conditions while the globin proteins were essentially unresponsive to the same drying treatments. Both isolates are excellent emulsifiers and good foaming agents under optimum conditions of protein concentration and pH.

PROTEIN-LIPID FILMS AS MEAT SUBSTITUTES. L.C. WU & R.P. BATES. *J. Food Sci.* **40**, 160–163 (1975)–Techniques have been developed for the formation, recovery and utilization of protein-lipid films. Manipulation of the films derived from a variety of pure and mixed protein-lipid systems–soybean, soy protein isolate, peanut, glandless cottonseed and milk–resulted in textured, high protein food ingredients suitable as meat extenders and substitutes. Important parameters influencing the organoleptic properties of the film products are protein source and content, protein:lipid ratio, moisture content, pressure, temperature and film alignment during fabrication. By varying conditions, a range of formulated products possessing organoleptic properties similar to animal protein foods can be produced. Fabrication of film foods represents an alternative process for producing textured protein foods.

KINETIC ANALYSIS OF LIGHT-INDUCED RIBOFLAVIN LOSS IN WHOLE MILK. R.P. SINGH, D.R. HELDMAN & J.R. KIRK. *J. Food Sci.* **40**, 164–167 (1975)–The deterioration of the quality of a liquid food during storage is influenced by several factors including light intensity, storage temperature and type of container. The objective of this investigation was to demonstrate the use of kinetic analysis in the establishment of the optimum storage conditions for whole milk. The riboflavin content of whole milk was monitored during storage in glass bottles, paper containers and various types of blow-molded plastic bottles. The storage conditions included 1.7, 4.4, and 10°C, and 150, 300 and 450 ft-c of light intensity. Containers were arranged in the storage room so that only one side and the top were exposed to the indicated light conditions. The riboflavin content was determined at 0, 24, 48 and 72 hr of exposure to each storage condition. Rate constants, which described the rate of riboflavin loss in the milk at each temperature, light intensity and container type, were determined using a numerical fitting analysis assuming first-order reaction. In general, the magnitude of rate constants indicated that riboflavin loss was greatest in glass or regular (translucent) plastic containers at the highest light intensities and temperatures. The influence of temperature on rate of riboflavin loss was investigated by evaluating activation energies. This parameter appears to be a good indicator of the container's ability to control light-induced vitamin loss in the product.

MASS PRODUCTION OF Rhizopus oligosporus SPORES AND THEIR APPLICATION IN TEMPEH FERMENTATION. H.L. WANG, E.W. SWAIN & C.W. HESSELTINE. *J. Food Sci.* **40**, 168–170 (1974)–Tempeh, a popular Indonesian soybean food made with *Rhizopus oligosporus*, and tempeh-like products made from other cereal grains have a mild, pleasant flavor and a potential for use in high-protein snacks. To facilitate tempeh fermentation, attempts were made to develop a suitable inoculum. Freeze-dried *R. oligosporus* spore preparations were made by fermenting rice, rice-wheat bran or wheat-wheat bran at a 40% moisture level for 4–5 days at 32°C. After freeze drying the spore preparations had viable spore counts of 10^7 per g, and viability did not change significantly after 6 months of storage at 4°C. At a level of 10^6 spores per 100g of cooked soybeans or pearled wheat, tempeh fermentations were successfully carried out in petri dishes, trays and plastic bags. The inoculated beans packed in plastic bags were frozen and fermented later as needed. Either the water-insoluble fraction of soybeans or the residue from soybean milk and tofu production served as good substrates for tempeh fermentation. Tempeh made from the residue had a texture and flavor similar to French-fried potatoes. Tempeh fermentation, therefore, is an excellent way to use this residue, considered a waste.

REMOVAL OF THRIPS DURING ASPARAGUS WASHING. R.C. RILEY, D.J. PROSTAK, J.P. REED & R.D. SNEE. *J. Food Sci.* **40**, 171–174 (1975)–Thrips on harvested asparagus spears can be substantially reduced by employing certain in-plant washing procedures. Our investigations have shown that increasing the soak water temperature from 21° to 71°C, adding 40–80 ppm pyrethrins, and increasing the spray rinse pressure from 25 to 60 psi significantly reduced the number of thrips on washed asparagus. Hot soak water opens the asparagus heads and pyrethrins irritate and activate thrips to enhance their removal.

REFRIGERATED BULK STORAGE OF CRANBERRY PUREE. B.G. SWANSON & K.G. WECKEL. *J. Food Sci.* **40**, 175–177 (1975)–Long term refrigerated bulk storage of cranberry puree for subsequent processing appears promising. The consistency, color, pH, titratable acidity and product capability of cranberry puree changed little during 22 wk of bulk storage at 4°C. Although cranberry puree with an initial pH of 2.70, soluble solids content of 7.0% and total solids of 8.80% supported mold and yeast growth, growth of molds and yeasts was arrested with 0.1% sorbic acid. Mean flavor scores of strained cranberry sauces prepared from cranberry puree after 22 wk of storage ranged from 3.6–5.8 on a 9.0 scale. The flavor scores indicate cranberry sauces prepared from cranberry puree held in bulk containers for long periods are not undesirable.

ASCORBIC ACID RETENTION IN ORANGE JUICE AS RELATED TO CONTAINER TYPE. O.W. BISSETT & R.E. BERRY. *J. Food Sci.* **40**, 178–180 (1975)–Single-strength orange juice (SSO) and frozen concentrated orange juice (FCOJ) stored in different containers at different temperatures were analyzed for ascorbic acid (AA) retention. SSOJ was studied in glass, polyethylene and polystyrene bottles and wax-coated cardboard cartons. AA reduction was fairly rapid (80% in 3–4 wk) in plastic and cardboard while glass afforded best (90% or better) retention. FCOJ was studied in foil-lined cardboard rectangular cartons and polyethylene-lined fiber cylindrical cans. Both retained 90% or more AA after 1 yr when the product remained frozen. Neither container was effective above freezing because of microbial spoilage. Juices reconstituted from frozen concentrates in containers used in the home indicated keeping juice cold is as important as type dispenser or container. Generally, each product retained 85% or more of the initial AA for the storage times and conditions usually recommended for that product.

A RAPID MICRO TECHNIQUE FOR TESTING BIODEGRADABILITY OF NYLONS AND RELATED POLYAMIDES. D.M. ENNIS & A. KRAMER. *J. Food Sci.* **40**, 181–185 (1975)–Nylons have excellent properties as food packages, but are not biodegradable. As a first step in modifying polyamides to retain existing properties, but also to be bio-

degradable, it was necessary to develop the following procedure which requires as little as 20 mg of test sample. Differences in respiration rates at 30°C of a mixed culture of soil microorganisms in the presence and absence of the test material serves as measure of biodegradability. Results with monomers, dimers, trimers and irradiated and nonirradiated polyamides are presented, including nylon 6,6; nylon 3/4/6; nylon 2/6 and an irradiated polymer of 2,4-pentane diamine and adipyl chloride. Biodegradability was detected after 2–13 days.

FORMULAE FOR PREDICTING GAS EXCHANGE OF FRESH PRODUCE IN POLYMERIC FILM PACKAGE. K. HAYAKAWA, Y.S. HENIG & S.G. GILBERT. *J. Food Sci.* **40**, 186–191 (1975)–Analytical formulae are obtained for predicting transient state oxygen and carbon dioxide concentrations in the microatmosphere of post climacteric fresh produce package. To obtain these formulae, it is assumed that the oxygen consumption rate or carbon dioxide evolution rate of fresh produce is affected by both oxygen and carbon dioxide concentrations in the surrounding atmosphere. From the analytical formulae simple algebraic equations are obtained. These equations are for predicting equilibrium oxygen and carbon dioxide concentrations in the microatmosphere of a package and also for predicting time after packaging at which it reaches steady gas exchange (equilibrium state time value). The algebraic equations are applicable when the oxygen consumption rate and carbon dioxide evolution rate of fresh produce are influenced only by oxygen and carbon dioxide concentrations in surrounding atmosphere, respectively. The equations are used for estimating equilibrium oxygen and carbon dioxide concentrations in fresh tomato and fresh banana packages. There is fair agreement between analytically estimated and experimentally determined concentrations. Equilibrium time values for the oxygen and carbon dioxide gas exchanges of the same sample packages are also calculated by the algebraic equations. The time values estimated are then compared with those determined through experimentation. There is fair agreement in the equilibrium time values for oxygen gas exchange. However, there is poor agreement in those values for carbon dioxide gas exchange. Through the regression analysis of data on transient state oxygen and carbon dioxide concentrations in the sample packages, it is concluded that this poor agreement is likely caused by the assumption, which is imposed for the experimental determination of respiration rate constants. The analytical formulae derived will be greatly useful for the optimization of packing parameters, since these parameters are explicitly included.

IMPROVEMENT OF SHELF LIFE OF PARTIALLY DEFATTED PEANUTS BY INTROMISSION OF NITROGEN INTO THE INTERSTICES OF THE PEANUTS. J. POMINSKI, H.M. PEARCE JR., H.L.E. VIX & J.J. SPADARO. *J. Food Sci.* **40**, 192–194 (1975)–Two procedures were devised to improve the shelf life of partially defatted peanuts (over 50% oil removed) by introducing nitrogen into the porous interstices of the defatted peanuts after roasting. One method consisted of cooling oil-roasted defatted peanuts in nitrogen and holding the peanuts under nitrogen until canned under vacuum; and the second method by cooling the oil-roasted defatted peanuts in air and holding the peanuts under nitrogen until canned under vacuum. Shelf-life studies showed that partially defatted peanuts with nitrogen in the interstices have longer shelf life than those containing air.

EFFECT OF LOW LEVEL GAMMA IRRADIATION ON GROWTH AND PATULIN PRODUCTION BY Penicillium patulum. L.B. BULLERMAN & T.E. HARTUNG. *J. Food Sci.* **40**, 195–196 (1975)–Spores as well as vegetative mycelia of two strains of *Penicilliumm patulum* were irradiated at 100 and 200 Krad to determine the effects on growth and patulin production. Irradiation of spores reduced subsequent growth in potato dextrose broth by both strains. Spores of one strain were more resistant to irradiation than spores of the second strain. Irradiation of mycelia resulted in a variable growth response in subsequent cultures in potato dextrose broth. Cultures grown from irradiated spores and mycelia produced less patulin than nonirradiated control cultures. There was a marked reduction in the amount of patulin produced per mg of dry mycelia with both strains when grown from either irradiated spores or mycelia.

EFFECT OF IRRADIATION ON VOLATILE CONSTITUENTS OF STORED HADDOCK FLESH. P. ANGELINI, C. MERRITT JR., J.M. MENDELSOHN & F.J. KING. *J. Food Sci.* **40**, 197–199 (1975)–The effects of irradiation on the volatile constituents found in haddock flesh following storage have been studied. Fresh haddock fillets were irradiated in sealed mylar bags at doses of 0, 0.2, 2.8 and 5.6 Mrad. The volatile constituents were collected from the samples by low temperature-high vacuum distillation technique and analyzed by combined gas chromatograph/mass spectrometry. All lots of haddock were sampled before storage at 5.5°C. The nonirradiated samples were stored for 14 days and irradiated samples for 30 days. The results show a substantial increase in the amounts of volatile components for the nonirradiated stored samples. Sulfur compounds (especially methyl mercaptan, dimethyl sulfide, and dimethyl disulfide), carbonyl compounds and trimethylamine increased greatly on storage of nonirradiated haddock. Haddock samples irradiated at 0.2 Mrad also show an increase in amounts of volatiles on storage. The haddock samples irradiated at both 2.8 and 5.6 Mrad show smaller amounts of volatile components after 30 days of storage. Benzene and toluene, although minor constituents in all samples, were present in larger quantities in the irradiated samples.

EFFECT OF TEMPERATURE AND HEATING TIME ON THE DETECTION OF OFF-FLAVOR IN AVOCADO PASTE. R. GARCÍA, J. ANDRADE & C. RCLZ. *J. Food Sci.* **40**, 200 (1975)–The appearance of off-flavor when avocado pulp is heated above a certain temperature has hindered its preservation by thermal processing. The effect of temperature and heating time on the appearance of off-flavor in an avocado paste prepared with fruits of the Aztec variety, spices and lime juice, was measured by sensory evaluation in triangular tests. The samples were heated in sealed 28g capacity polyethylene bags. A curve with a preliminary z value of 24.26 was obtained. It can be used in combination with a TDT curve of the appropriate microorganism to determine the optimum conditions for a high temperature-short time process.

POLYGALACTURONASE ACTIVITY IN CITRUS FRUIT. J. RIOV. *J. Food Sci.* **40**, 201–202 (1975)–Based on recent work and literature data, work was designed to study the possible factors which might interfere with the determination of polygalacturonase (PG) in citrus fruit tissues and to determine whether PG occurs in citrus fruit. No inhibitory effect of citrus extracts on pectinase activity was detected in the PG preparations used. PG activity was found in flavedo, albedo and pulp of Shamouti and Valencia oranges, grapefruit and lemons. Except for grapefruit, the highest activity, on a fresh weight basis, was found in the flavedo and the lowest activity in the pulp. In grapefruit, the highest activity was found in the albedo. The effect of ethylene on PG activity in citrus fruit tissues was tested by treating Valencia oranges and grapefruit with 10 μl/liter ethylene for 48 hr and found to have no effect compared to air controls. However, ethylene was found to increase PG activity in the abscission zones of leaves and fruit of citrus.

VISCOSITY OF MANGO NECTAR AS RELATED TO PECTIC SUBSTANCES. A.R. SAEED, A.H. El TINAY & A.H. KHATTAB. *J. Food Sci.* **40**, 203–204 (1975)–The relationship between harvest date, pectin content of hard and ripe mango fruit and their effect on viscosity of mango nectar was investigated. Total pectin was found to decrease as the harvest season advanced in the cases of Kitchener and Alphonso. Total pectin decreased after ripening the fruits in the laboratory. Water soluble pectin varied between trace to nil in the hard fruits, while it increased in the ripe fruits. Pectic substances were found to have an effect on the viscosity of mango nectar.

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CHARACTERIZATION OF PECTIC SUBSTANCES IN MANGO MARC. A.R. SAEED, A.H. El TINAY & A.H. KHATTAB. *J. Food Sci.* **40,** 205–206 (1975)–Characterization of pectic substances in mango marc showed that there were differences in the equivalent weight, free and esterified carboxyl group, degree of esterification and AUA among the three varieties. Weight average molecular weight was 1.6×10^4, 2.1×10^4 and 2.7×10^4 for Alphonso, Kitchener and Abu Samaka, respectively. The intrinsic viscosity increased as the molecular weight increased showing that Abu Samaka has better pectin quality followed by Kitchener then Alphonso.

VOLATILE COMPONENTS OF ROASTED MACADAMIA NUTS. W.O. CRAIN JR. & C.S. TANG. *J. Food Sci.* **40,** 207–208 (1975)–Volatile compounds from ground, freshly roasted macadamia nuts were collected by vacuum techniques. The concentrated essence was separated into neutral and basic fractions and the individual components were identified or tentatively identified by their GC retention indices and mass spectra. Similar to other roasted nuts, the neutral fraction of the volatile consists mainly of alcohols, aldehydes and ketones, and the basic fraction a series of pyrazines. Methyl sulfide was the sole major constituent present in the headspace of macadamias not reported in peanuts, filberts and pecans.

PREVENTION OF SKIN SHRINKAGE IN CUT-UP HOT-PACKED BROILERS. T.C. CHEN & A.S. ARAFA. *J. Food Sci.* **40,** 209–210 (1975)–The cutting of "hot-packed" broilers resulted in a reduced eye appeal of the product due to the severe skin shrinkage. Greatest shrinkage of the skin was observed on the ventral and the knee regions, while the lowest readings were recorded from the dorsal area. Skin shrinkage took place instantly after cutting and remained essentially the same throughout the entire refrigerated storage period. A method involving rapid air chilling to lower the surface temperature before cutting was developed to reduce the skin shrinkage of the cut-up broilers. As the subcutaneous temperature of hot-packed carcasses was reduced, skin shrinkage was reduced. When the temperature was reduced to 1.1°C, shrinkage ceased.

MATHEMATICAL ANALYSIS OF SOLUBILIZATION KINETICS AND DIFFUSION IN FOODS. H.G. SCHWARTZBERG. *J. Food Sci.* **40,** 211–213 (1975)–Mathematical techniques are presented for determining reaction rate constants and solid-substrate diffusivities for first-order solubilization reactions in foods. It is shown that reaction rate constants cannot be correctly determined by the usual solubilization tests unless suitably small particles are used. A parameter which identifies a suitably small particle size and which indicates when solubles recovery can be speeded up by finer grinding is derived.

NATURALLY OCCURRING TOXICANTS IN FOODS. A Scientific Status Summary. IFT EXPERT PANEL ON FOOD SAFETY & NUTRITION. *J. Food Sci.* **40,** 215–222 (1975)–The natural chemical components of foods constitute the greatest amount and widest variety of chemical substances consumed by man over his lifetime; therefore, a comprehensive consideration of toxicology of natural chemical components of foods should take into account the total picture of foods as the most complex part of man's chemical environment. Sources of both natural and man-made toxic chemicals in foods are reviewed, followed by a comprehensive discussion of many aspects of the subject (e.g., toxicologic interactions, margins of safety, benefits vs. risks, etc.). It is concluded that viewing all chemicals present in our food supply in perspective – natural components, agricultural chemicals, food additives, and natural man-made contaminants – it is clear that the greatest area of the unknown involves the normal and natural components of our foods. Thus to achieve a more appropriate balance in evaluating food safety in general, equal attention should be given to these substances as is given to additives and pesticide residues – as well as the simultaneous presence of both groups of chemicals and their resulting chemical and toxicologic interactions. As far as man's food intake is concerned, the ultimate goal is to understand not only what constitutes ultimate nutritional content but also what involves minimum long-range, lifetime toxicologic hazards in the diet.

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A SYMPOSIUM . . . complete in this issue

NUTRITIONAL PERSPECTIVES AND ATHEROSCLEROSIS

INTRODUCTION

RELATIONSHIPS between nutrition, atherosclerosis and coronary heart disease have been the subject of intense interest for the past 15 years. Epidemiologic and clinical investigations have identified a number of risk factors associated with coronary heart disease. These include an elevation of plasma lipids, especially cholesterol, but also triglycerides. Other risk factors include high blood pressure, heavy cigarette smoking, obesity, physical inactivity and genetics. The risk of developing coronary heart disease is positively correlated with the concentration of cholesterol in plasma. It appears to be relatively small at levels of less than 220 mg/100 ml but many American men and women maintain levels above this when consuming their usual diets. Extensive evidence indicates that the level of cholesterol can be lowered in most persons by appropriate dietary modifications such as reduction of total dietary fat, substitution of polyunsaturated fatty acids for saturated fat and reduction in the consumption of foods rich in cholesterol. Less is known about plasma triglycerides but they can be modified also by dietary intervention.

The Council on Foods and Nutrition of the American Medical Association in a recent statement on Diet and Coronary Heart Disease has made the following points:

(1) Measurement of the plasma lipid profile, particularly cholesterol, should become a routine part of all health maintenance examinations;

(2) Persons falling in a "risk category" on the basis of their plasma lipid levels should be made aware of this and receive appropriate dietary advice;

(3) Care should be taken that the dietary regimen does not compromise the intake of essential nutrients;

(4) Since the above recommendations will be effective only if they can be accomplished with relative ease, modified and ordinary foods useful for this purpose should be readily available on the market; and

(5) "High priority should be given to the conduct of studies that will determine reliably the extent to which the modification of plasma lipids, by dietary or other means, as well as modification of other risk factors, can reduce the risk of developing coronary artery disease" (AMA, 1972).

This last recommendation should receive emphasis. We still do not have incontrovertible data on the effectiveness of dietary modifications in the prevention of coronary artery disease.

In the symposium today, some recent investigations of relationships between various dietary constituents, lipids and atherosclerosis will be presented. The first paper by Jones et al. (1975) deals with plasma cholesterol concentrations in monkeys as influenced by diet and phenotype. Kritchevsky et al. (1975) discuss the relationship of diet to lipid metabolism, specifically the integral part that fiber, a nonnutritive component of the diet, plays. Kummerow (1975) presents evidence of the possible role of lipids in the development of atherosclerosis. Carroll and Hamilton (1975) conclude the symposium with a discussion of the effects of dietary protein and carbohydrate on plasma cholesterol levels in relation to atherosclerosis.

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SYMPOSIUM: Nutritional Perspectives and Atherosclerosis

PLASMA CHOLESTEROL CONCENTRATIONS IN SQUIRREL MONKEYS AS INFLUENCED BY DIET AND PHENOTYPE

INTRODUCTION

THE SQUIRREL MONKEY (*Saimiri sciureus*) offers an interesting animal model for studies on the regulation of plasma cholesterol concentrations. We have previously reported that certain individual animals, hyporesponders (HO), are able to maintain near normal plasma cholesterol concentrations when fed diets containing significant amounts of cholesterol; other individuals, hyperresponders (HP), become hypercholesterolemic when fed atherogenic diets and develop extensive atherosclerosis (Clarkson and Lofland, 1970; Lofland et al., 1970, 1971). From selective breeding experiments, we have demonstrated a high degree of genetic control over plasma cholesterol homeostasis (Clarkson et al., 1971). When the term "phenotype" is used herein, it refers to the plasma cholesterol response described above. From these experiments, however, it was clear that the composition of the diet, irrespective of its cholesterol content, also exerted an important influence on the ultimate concentration of plasma cholesterol attained by any individual animal.

In the present studies, we have attempted to identify the mechanism(s) involved in both the genetic and dietary regulation of plasma cholesterol concentration. Included in the mechanisms which may be involved are: (a) regulation of cholesterol absorption; and (b) control of the excretion of cholesterol and/or bile acid. In the experiments described here, we have measured these factors in HP and HO squirrel monkeys fed diets containing cholesterol and either butter or safflower oil as the source of dietary fat. A two by two factorial design was used in order to allow us to assess both genetic and dietary effects in four animals of either phenotype fed either of the two diets.

MATERIALS & METHODS

Animals and diets

Forty adult male squirrel monkeys of the Brazilian type were obtained from the Tarpon Zoo, Tarpon Springs, Fla. Initially, the animals were fed our standard atherogenic diet (MacNintch et al., 1967) in which lard is the source of dietary fat and the cholesterol concentration is 1 mg/cal. After 1, 2 and 3 months of feeding, blood samples were obtained from the femoral vein for the measurement of plasma cholesterol concentrations, using an automated method (Block et al., 1966). On the basis of the response of the animals to the diet, eight animals were chosen for further studies, four HO and four HP. The mean plasma cholesterol concentrations of the four HO during this period were 275, 301, 274 and 247 mg/dl, while the four HP averaged 594, 646, 1054 and 548 mg/dl. These animals were fed Special Purina Monkey Chow-25 (Ralston-Purina Co., St. Louis, Mo.) for 3 months before feeding the liquid diets described below.

During the subsequent months of the experiment, the animals were housed individually in our primate "metabolic ward" in stainless steel metabolism cages measuring 46 cm wide by 61 cm deep by 81 cm high. From these cages complete fecal collections were made. A baffle device prevented the liquid formula diet from being mixed with the feces.

It was our purpose to study the influence of two dietary fats differing widely in unsaturation of fatty acids. Accordingly, a liquid formula diet was prepared (Table 1) in 16 kg batches, using single lots of butter and safflower oil. Since safflower oil contains plant sterols and butter contains cholesterol, the fats were assayed for these substances by gas-liquid chromatography (GLC) and these components were equalized in the two diets by adding either β-sitosterol or cholesterol. The animals were allowed *ad libitum* access to the diets between the hours of 8 a.m. and 4 p.m. The amounts of diet consumed were measured daily by weighing the bottles before and after feeding. Two HO and two HP were fed each diet.

Table 1—Composition of the liquid formula diets[a]

Non-fat dry milk solids	1680g
Complete vitamine mixture[b]	120g
Salts mixture, USP XIV	120g
Sucrose	1200g
Gelatin	80g
Safflower oil *or*	890 ml
Butter	988g
Cholesterol (when added):	
to butter diet	11.5g
to safflower oil diet	13.9g
β-sitosterol (butter diet only)	2.4g
Water	12 liters

[a] Cholesterol and β-sitosterol were dissolved in the warmed fats and blended with the other ingredients to provide a stable emulsion.

[b] Complete Vitamin Fortification Mixture formula from Nutritional Biochemicals Corp., Cleveland, Ohio, with vitamin D_3 substituted for D_2. Caloric value: 1.14 cal/g; Cholesterol content: 0.13 mg/cal (basal); 0.75 mg/cal (test).

The animals were studied during two periods: the first was a 7-wk interval during which the animals consumed the basal formula containing only the amount of cholesterol present in butter (low cholesterol diet, with 0.13 mg cholesterol/cal of diet). Two 3-day fecal collections were made during this period. Feces were collected and frozen daily for 3 days, and pooled for the 3-day fecal collection. At the end of 53 days, the animals were fed the same diet, except that crystalline cholesterol (dissolved by warming in the fat) was added to a final concentration of 0.75 mg/cal (high cholesterol diet). During the next 3 months, eight additional 3-day fecal collections were made, as described above.

Sterol balance studies

We have described elsewhere our techniques for conducting sterol balance studies on nonhuman primates (Lofland et al., 1972) based on a combination of isotopic and GLC methods (Grundy and Ahrens, 1969). In brief, they consist of the intravenous administration of isotopically-labeled cholesterol at the start of the low cholesterol feeding period (in the present experiments, each animal received a single injection of 85 μC of cholesterol-1,2-^{3}H, prepared as an emulsion in saline, ethanol and Tween 20). The pooled feces from a 3-day collection period were weighed and homogenized, and cholic acid-26-^{14}C was added as an internal standard (I.S.) for the acidic steroid fraction (recovery of cholic acid I.S. was greater than 90%). Neutral and acidic steroids were separated by solvent extraction, purified by thin-layer chromatography (TLC), and, in the case of neutral steroids, quantified by GLC. Acidic steroids were quantified by the radioactivity (^{3}H) recovered in that fraction from the feces. The specific activity of cholesterol-1,2-^{3}H in the plasma at the midpoint of the fecal collection period was used to calculate the mg bile acid excreted. Radioactivity measurements were made using a Beckman DPM-100 liquid scintillation spectrometer (Beckman Instruments, Fullerton, Calif.). Samples were dissolved in 10 ml of a solution of 6g of diphenyloxazole per liter of toluene, and were counted to a 2-sigma error of < 3%. Correction for quenching was done using an external standard-channels ratio method. Radioactive materials were obtained from New England

Nuclear Corp., Boston, Mass., and were checked for purity by TLC before use. At each interval where a fecal collection was made, a sample of each diet being consumed at that time was assayed for cholesterol content by the same techniques as used for feces. Our previous studies (Lofland et al., 1972) showed that in the squirrel monkey there is essentially no sterol ring degradation in the gut during digestion. All samples were run in duplicate and were repeated if duplicates did not agree within 5%.

In the present studies, as before (Lofland et al., 1972), we calculated absorption as the difference between unabsorbed dietary cholesterol and the amount known to have been ingested. Excretion was calculated as the sum of the neutral and acidic steroid fractions. As previously observed, however, when one attempts to calculate cholesterol synthesis as the difference between excretion and absorption, negative values are obtained when animals are not in the steady state, indicating that body cholesterol pools are expanding. Accordingly, the present experiments yield no information regarding rates of synthesis. The absorption and excretion data, however, should be valid even for the fecal collections taken within a week after the start of the high cholesterol feeding. These calculations do not rely on the isotopic die-away curves. They do assume a rapid (1–2 day) equilibration of the specific activity of cholesterol and bile acids in the bile.

RESULTS

HP and HO squirrel monkeys on butter diet

Within 30 days after the start of the high cholesterol-butter diet, the plasma cholesterol concentrations of the two HP monkeys had increased dramatically from 250 mg/dl to 750 mg/dl (Fig. 1 and 2). Cholesterol absorption also increased dramatically, and immediately after the start of the high cholesterol-butter feeding (Fig. 1 and 2). Qualitatively, changes in plasma cholesterol concentrations appeared to parallel the changes in cholesterol absorption. Bile acid excretion increased moderately in one monkey (Fig. 1) and somewhat less in the other (Fig. 2) in the same 30-day feeding period. Seventy days after the start of the high cholesterol-butter diet, however, bile acid excretion increased in both animals. Cholesterol excretion remained essentially unchanged during the entire feeding period.

The two HO monkeys on the same high cholesterol-butter diet (Fig. 3 and 4) had absorption and excretion patterns similar to the two HP monkeys just described. A large increase in cholesterol absorption by monkey 1722 (Fig. 3) occurred immediately after the start of the test diet feeding, and the plasma cholesterol concentration rose to a maximum of 750 mg/dl by 45 days of cholesterol feeding. Monkey 1748 (Fig. 4) had the lowest amount of cholesterol absorbed per day of the four monkeys just described, as well as the smallest increase in plasma cholesterol. Changes in plasma cholesterol concentrations paralleled cholesterol absorption changes in the two HO, just as in the two HP. This is especially apparent in the last three collection periods shown in Figure 3. Bile acid excretion again did not increase dramatically until 55 days of high cholesterol-butter diet feeding, with maximum excretion at 70 days (Fig. 3 and 4) (this increase could have occurred sooner, sometime between 35 and 55 days). Cholesterol excretion likewise increased only slightly (Fig. 3) or not at all (Fig. 4).

HP and HO squirrel monkeys on safflower oil diet

The absorption and excretion patterns for the four monkeys ingesting the safflower oil diet are similar to, but less dra-

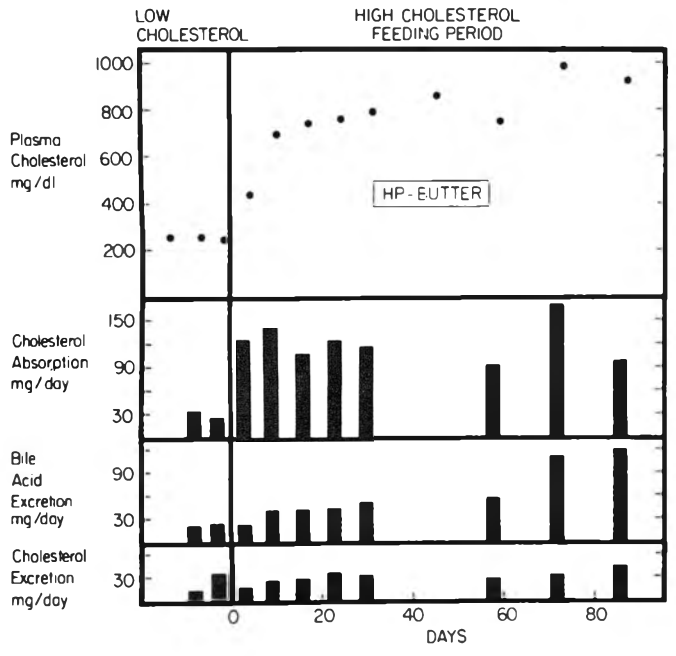

Fig. 1—Changes in plasma cholesterol concentration, cholesterol absorption and excretion in a HP squirrel monkey consuming a butter-cholesterol diet. This monkey (no. 1708) was fed a low cholesterol (0.13 mg/cal) diet, with butter as 40% of calaries, prior to day 0. After day 0 it was fed a high cholesterol (0.75 mg/cal) diet with butter as 40% of calories for 100 days. Cholesterol absorption and excretion, and bile acid excretion, were measured from ten fecal samples collected during the low and high cholesterol feeding periods.

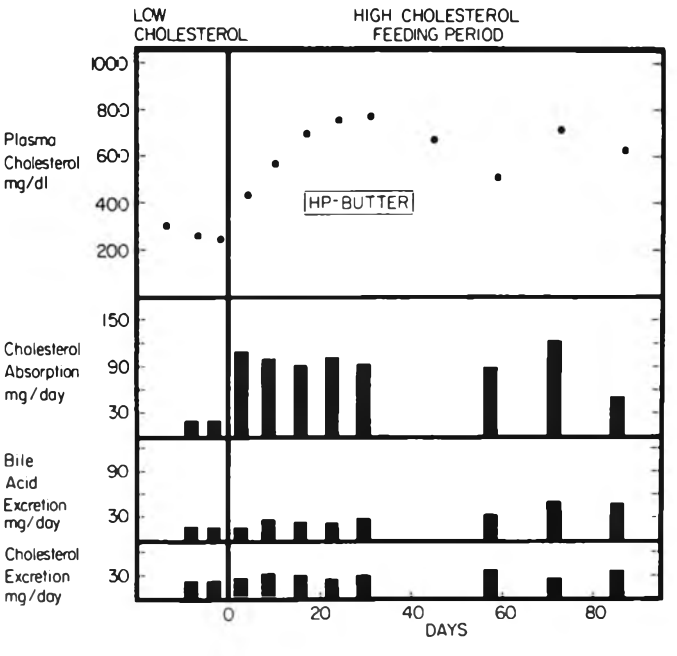

Fig. 2—Changes in plasma cholesterol concentration, cholesterol absorption and excretion in a HP squirrel monkey (no. 1728) consuming a butter-cholesterol diet. See Fig. 1 for experimental details.

matic than, the patterns for the monkeys ingesting the butter diet. One of the HP monkeys which demonstrated a moderate increase in cholesterol absorption had a moderate increase in plasma cholesterol concentration (#1711, Fig. 5). The cholesterol absorption of each of the other three monkeys averaged about 50 mg/day, which was the lowest of any of the eight monkeys. Increases in plasma cholesterol concentrations were also minimal or absent in these three monkeys (Fig. 6–8). Bile acid excretion did not increase at all in the first 30 days of the test diet for any of the monkeys consuming saf-

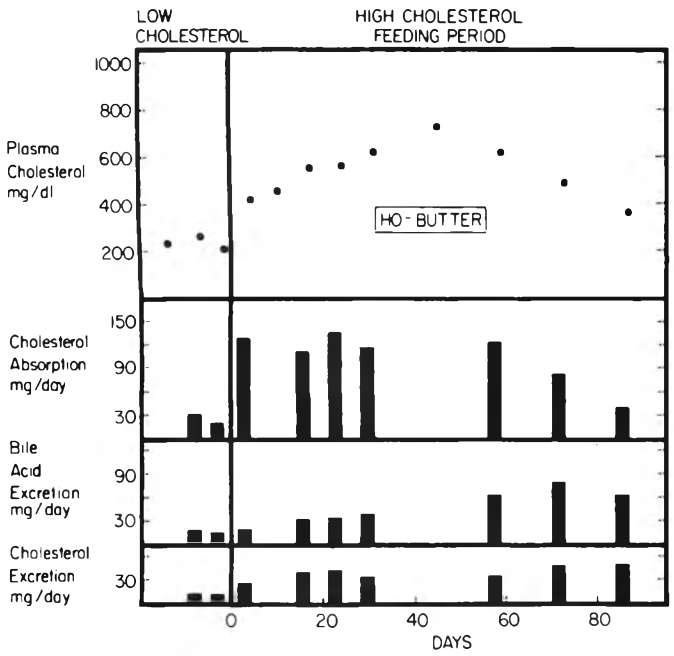

Fig. 3—Changes in plasma cholesterol concentration, cholesterol absorption and excretion in a HO squirrel monkey (no. 1722) consuming a butter-cholesterol diet. See Figure 1 for experimental details.

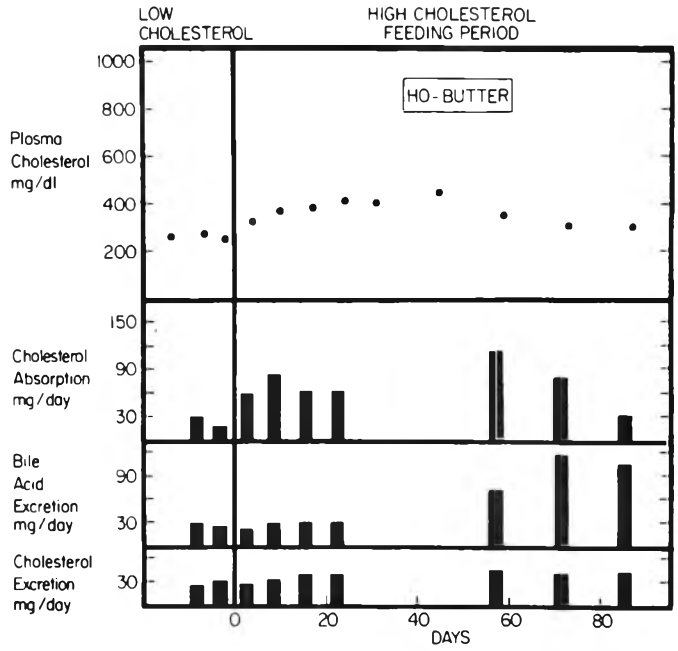

Fig. 4—Changes in plasma cholesterol concentration, cholesterol absorption and excretion in a HO squirrel monkey (no. 1748) consuming a butter-cholesterol diet. See Figure 1 for experimental details.

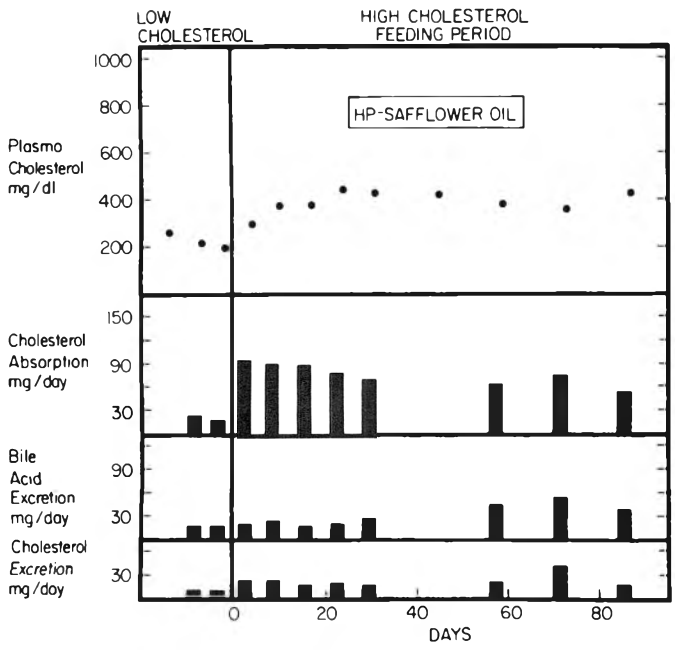

Fig. 5—Changes in plasma cholesterol concentration, cholesterol absorption and excretion in a HP squirrel monkey (no. 1711) consuming a safflower oil-cholesterol diet. Safflower oil was 40% of calories. See Figure 1 for experimental details.

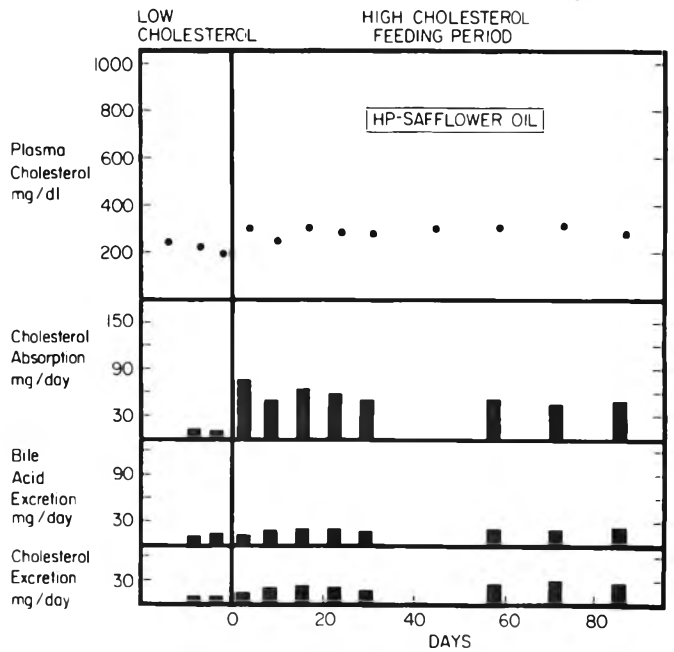

Fig. 6—Changes in plasma cholesterol concentration, cholesterol absorption and excretion in a HP squirrel monkey (no. 1724) consuming a safflower oil-cholesterol diet. See Figure 1 for experimental details.

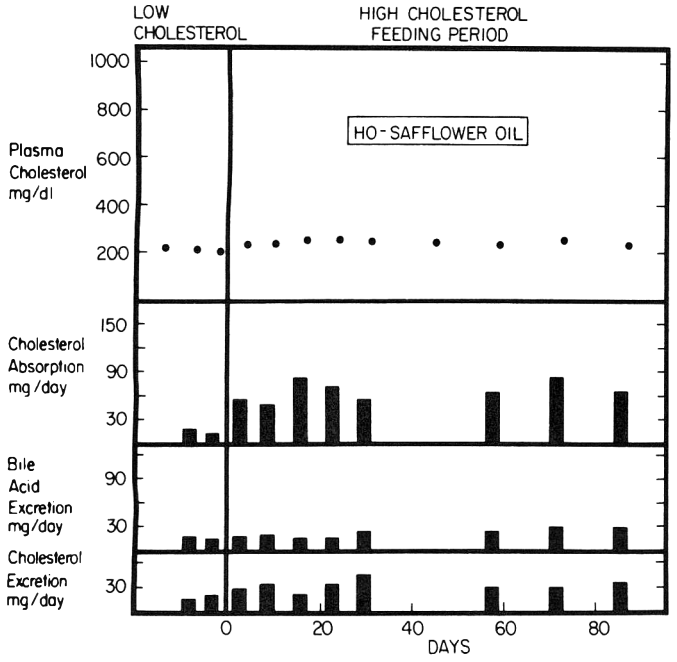

Fig. 7—Changes in plasma cholesterol concentration, cholesterol absorption and excretion in a HO squirrel monkey (no. 1707) consuming a safflower oil-cholesterol diet. See Figure 1 for experimental details.

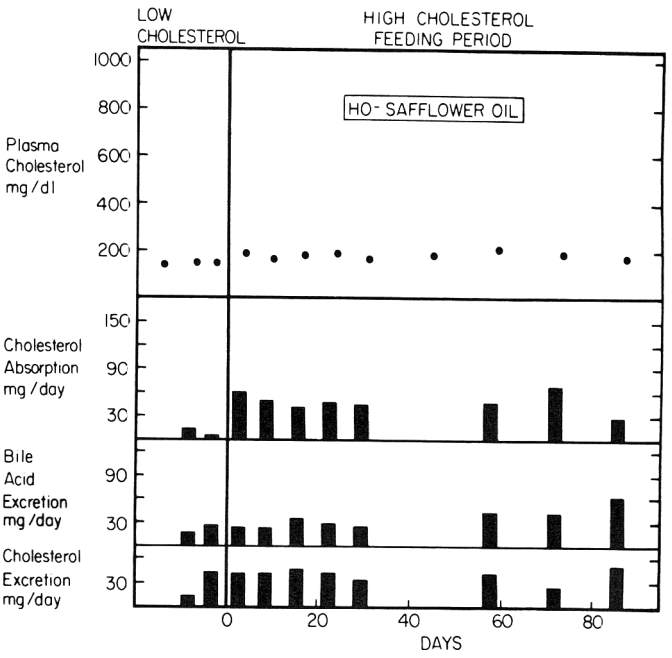

Fig. 8—Changes in plasma cholesterol concentration, cholesterol absorption and excretion in a HO squirrel monkey (no. 1717) consuming a safflower oil-cholesterol diet. See Figure 1 for experimental details.

flower oil. Bile acid excretion did increase moderately for one HP (Fig. 5) and one HO (Fig. 8) after 55 days of high cholesterol-safflower oil feeding. Cholesterol excretion was low and remained essentially unchanged for all four monkeys throughout the feeding period.

Phenotypic differences in per cent absorption of cholesterol

There were obvious differences in the amount of cholesterol absorbed by the eight squirrel monkeys (Fig. 1–8), but the cause of these differences was unclear. A monkey with a lower absorption could have (a) ingested less cholesterol or (b) absorbed a lower percentage of the cholesterol ingested. In order to determine the correct cause, we compared the absorption data from pairs of monkeys consuming nearly equal amounts of cholesterol per day. We also compared the absorption data by phenotype: one HP versus one HO without regard to kind of fat in the diet (Fig. 9).

Such comparisons revealed a lower per cent absorption of cholesterol for the HO in every pair. In the data shown in Figure 9A, for animals in which the amount of cholesterol ingested per day was quite similar, the HO (1748) absorbed only about one-half the amount of cholesterol absorbed by the HP (#1708). For the pair consuming the safflower oil-cholesterol diet (Fig. 9B), the cholesterol ingested was the same, while the amount absorbed was lower for the HO. When the data from a HP on the butter diet were compared with a HO on the safflower oil diet (Fig. 9C), the results were quite similar to those seen when both monkeys were fed the butter diet (Fig. 9A). In the fourth pair (Fig. 9D), where HO ingested more cholesterol, the HO still absorbed a smaller percentage of the ingested cholesterol than the HP.

The per cent absorption comparisons in Figure 9 are averages of the data from

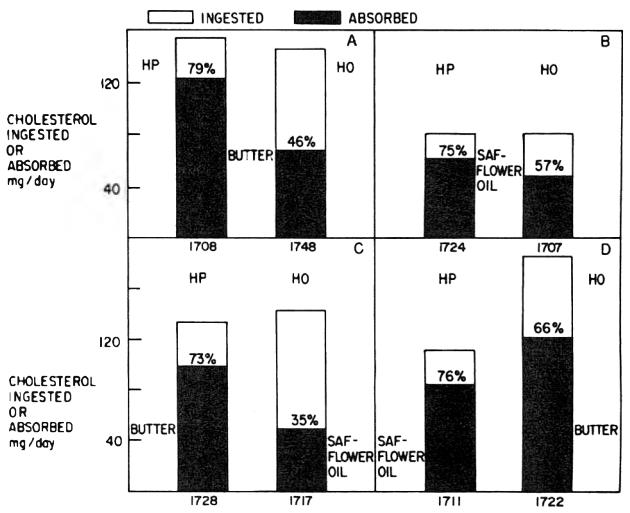

Fig. 9—A and B—Comparison of per cent absorption of cholesterol in HP and HO monkeys matched for similar cholesterol ingestion.

C and D—Comparison of per cent absorption in HP and HO monkeys consuming either a butter or safflower oil diet. In all cases, the data represent average values for five fecal collection periods made during the first month of high cholesterol feeding (0.75 mg chol/cal diet).

Table 2—Per cent absorption of cholesterol for last three collection periods[a]

Phenotype	Diet	Monkey #	Cholesterol ingested (mg/day)	Cholesterol absorbed (%)
HP	Butter	1708	159	75
HO	Butter	1748	182	40
HP	Safflower oil	1724	80	61
HO	Safflower oil	1707	128	65
HP	Butter	1728	131	65
HO	Safflower oil	1717	128	40
HP	Safflower oil	1711	90	73
HO	Butter	1722	182	44

[a] 55--90 days after the start of high cholesterol feeding

Table 3—Analysis of variance between phenotype and dietary fat for ingestion and per cent absorption of cholesterol

Variable	Source of variance	Sum of squares	Degrees of freedom	Variance	Variance ratio (F)[a]	Probability[b]
Average mg	HP(118) X HO(148)	13,912	1	13,912	5.857	N.S.[d]
cholesterol	Butter(159) X Saff. oil(107)	42,025	1	42,025	17.692	< 0.025
ingested	Interaction	47		47	0.020	N.S.
per day[c]	Between animals	9,501	4	2,375		
Average %	HP(73%) X HO(49%)	9,037	1	9,037	11.526	< 0.05
cholesterol	Butter(62%) X Staff. oil(61%)	28	1	28	0.036	N.S.
absorbed	Interaction	0.47	1	0.47	0.001	N.S.
per day[c]	Between animals	3,136	4	784		

[a] The variance ratio (F) equals variance of a given row divided by variance between animals.
[b] Probability that variance ratio could be as large, or larger, due to change alone
[c] Mean values in parentheses in second column. Average of all eight collections during the high cholesterol feeding period
[d] N.S. = not significant.

the first month of high cholesterol feeding (the first five collection periods). Per cent absorption averages were also calculated for the last three collection periods, which were 55–90 days after the start of high cholesterol feeding (Table 2). In three of the four pairs, the HO absorbed a lower per cent of the ingested cholesterol than did the HP. The per cent absorption of cholesterol (average of all eight collection periods) was significantly lower for HO than for HP (Table 3). There was no significant difference in the amounts of cholesterol ingested between the two phenotypes (Table 3).

Dietary fat differences in ingestion of cholesterol

Although there was no significant difference in per cent absorption between the *butter and safflower* oil diets, those monkeys fed the butter diet consumed significantly more diet than those monkeys fed the safflower oil diet (Table 3).

DISCUSSION

IT IS RECOGNIZED that the number of animals used in these experiments is small, due to the difficult nature of conducting sterol balance studies in nonhuman primates. We feel, however, that the data permit certain tentative conclusions.

Results of these experiments indicate that the phenotypic difference between HO and HP squirrel monkeys may be, at least in part, due to differences in absorption of dietary cholesterol. The increases in cholesterol absorption were observed to parallel the increases in plasma cholesterol, and the amount of cholesterol absorbed per day was higher for HP than for HO. One obvious cause for this difference could have been that the HP simply ingested more cholesterol per day than did the HO. The average cholesterol ingested per day for the HP, however, was not significantly different from the ingestion of the HO (Table 3). Another explanation is that the HP absorbed a higher percentage of the ingested cholesterol than did the HO. This possibility is supported by the results of these experiments, since we found the mean per cent absorption of cholesterol to be significantly higher for the HP (Table 3). This phenotypic effect on per cent absorption can also be seen when the data from a HP were matched with a HO which consumed similar amounts of cholesterol per day (Fig. 9 and Table 2). The HP absorbed a higher percentage of the dietary cholesterol in every pair but one, in which absorption was measured 55–90 days after the start of high cholesterol feeding (Table 2, monkeys 1724 and 1707). We do not know whether this phenotypic effect remains or disappears after periods of cholesterol feeding longer than 90 days. It has been found (Eggen and Strong, 1972) that six high-responding rhesus monkeys had significantly higher per cent absorption after 8 months of cholesterol feeding than did six low-responding rhesus monkeys. A difference in per cent absorption between HO and HP squirrel monkeys was also observed in an earlier experiment from this laboratory (Lofland et al., 1972). However, when one calculates the mean change in plasma cholesterol concentration per mg of cholesterol absorbed (Table 4), it is apparent that HP are more sensitive to a given amount of dietary cholesterol than are HO. The present studies do not, of course, explain this difference in sensitivity, which is probably related to simulataneous changes in relative rates of absorption, synthesis and excretion.

In contrast to the phenotypic effect, the dietary fat effect in these experiments

Table 4—Change in plasma cholesterol concentrations per mg of cholesterol absorbed

Phenotype	Diet	Monkey #	ΔPC/mg abolesterol absorbed[a]
HP	Butter	1708	3.6
		1728	3.8
HO	Butter	1722	2.7
		1748	1.6
HP	Safflower oil	1711	2.0
		1724	1.3
HO	Safflower oil	1707	0.30
		1717	0.60

[a] ΔPC = (mean plasma cholesterol during high cholesterol feeding) − (mean plasma cholesterol during low cholesterol feeding). Mg cholesterol absorbed = mean mg cholesterol during high cholesterol feeding.

appears to be related to the amount of cholesterol ingested. Those monkeys consuming cholesterol in the butter diet did absorb more cholesterol, and achieved higher plasma cholesterol concentrations (Fig. 1–8) than the safflower oil-fed monkeys. The butter-fed monkeys, however, ingested significantly more cholesterol per day than the safflower oil-fed group (Table 3). The per cent absorption of cholesterol from the two diets was the same (Table 3). It is possible that the butter diet was simply more palatable to the monkeys than the safflower oil diet.

Cholesterol excretion increased only slightly during the first month of high cholesterol feeding (Fig. 1–8). This suggests that there was little compensatory response by this pathway, a response which could be expected to decrease the concentration of cholesterol in the blood. Bile acid excretion increased to a significant degree only after 55–90 days of high cholesterol feeding (Fig. 1–8). Since we made no fecal collections between 35 and 55 days, the bile acid excretion could have increased somewhat earlier. Nevertheless, plasma cholesterol had reached maximum concentrations before the increases in bile acid excretion occurred. This suggests that the excretion of cholesterol via enhanced catabolism to bile acids occurs too late to prevent the initial plasma cholesterol increase. On the other hand, bile acid excretion may well have been involved in determining the eventual plasma cholesterol level in the steady state. This delayed response of increased bile acid excretion was also observed in our earlier study in which we (Lofland et al., 1972) found increased bile acid excretion in the last 7 wk of a 13-wk cholesterol feeding period, with the HO squirrel monkeys having significantly ($P < 0.05$) higher increases than the HP. We previously observed that squirrel monkeys lack the ability to control cholesterol homeostasis by decreasing their absorption with time. In the present experiments, this finding was less consistently observed.

We do not know the contribution of cholesterol synthesis to the increases seen in plasma cholesterol concentrations, since we were unable to measure synthesis in the nonsteady state. If synthesis had decreased more significantly or more rapidly in HO than in HP after the start of high cholesterol feeding, the total amount of cholesterol entering the blood would be less, and possibly would decrease the plasma cholesterol response. Alternatively, inhibition of cholesterol synthesis might have been more effective by cholesterol bound to a lipoprotein containing more unsaturated fat, such as safflower oil. Thus cholesterol synthesis could be a factor influencing the effects of both phenotype and diet.

There is some evidence from studies on man (Grundy and Ahrens, 1970) that cholesterol is redistributed from blood into other body pools more dramatically when the diet contains a more unsaturated fat. The present experiments shed no light on this aspect of sterol metabolism in monkeys.

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SYMPOSIUM: Nutritional Perspectives and Atherosclerosis
NONNUTRITIVE FIBER AND LIPID METABOLISM

THE MAIN THRUST of work on the relationship of diet to lipid metabolism has been, understandably, that of defining the nature and role of various dietary nutrients—lipids, carbohydrates and proteins. It appears now that a nonnutritive component of the diet, fiber, may play an integral part.

Role of fiber

The role of fiber in the etiology of certain types of cancer and in the development of ischemic heart disease was reviewed by Burkitt (1971) and Trowell (1972a, b, c). These reviews focused attention on earlier findings which had had no apparent unifying thread and have led to a redefinition of what constitutes fiber. In general, food tables report food carbohydrate as that amount of material remaining after the amounts of moisture, ash, fat and protein have been deducted. However, this designation encompasses a variety of chemical entities which behave differently under chemical (acid and base) extraction than towards the enzymes and bacteria of the digestive tract. Trowell (1972a, b, c) defines the structural polysaccharides and lignins present in the plant cell wall (elements which resist enzymic hydrolysis) as dietary fiber (as distinguished from crude fiber). Dietary fiber and the so-called "unavailable carbohydrate" may be identical (Southgate, 1969).

As so often happens, the introduction of a definition may serve as the focus for the reassessment of earlier results. This, in turn, can lead to a better definition of underlying mechanisms.

Influence of fat and roughage

In 1958, Lambert et al. reported that it was possible to induce atherosclerosis in rabbits with a cholesterol-free, semi-purified diet high in saturated fat. Feeding of saturated fat alone had been shown to be atherogenic in some cases and not in others. Collation of available data (Kritchevsky, 1964) showed that saturated fat alone was atherogenic when present in a semi-purified diet but never when added to laboratory chow (Table 1). At that time, it was noted that two of the major variables between the semi-purified and chow-based diets were the amount and type of carbohydrate and roughage. Subsequent work (Kritchevsky and Tepper, 1968) indicated that the ether-extracted residue of chow possessed some "protective" properties whereas the extract itself had none. It was later found that the type of carbohydrate did, indeed, also exert an effect on atherogenesis (Kritchevsky et al., 1968). Moore (1967) observed that varying the type of fiber in the atherogenic semisynthetic diet affected serum cholesterol levels and severity of atherosclerosis in rabbits (Table 2).

Effects of isocaloric, isogravic diets

In our studies on the effects of isocaloric, isogravic diets, we compared the absorption of cholesterol in rats fed such diets with rats fed chow (Kritchevsky et al., 1973). The diets contained 50% of calories as dextrose, casein, corn oil or

Table 1—Influence of fat on atherosclerosis in rabbits fed choiesterol-free diets

Fat Added	%	Duration (mo)	Atheroma	Reference
Added to chow:				
Cream	15–20	1.5–6	0.0	a
Crisco	9	2	0.1	b
Peanut meal	75	12	2/33	c
Coconut oil	12	3	0.0	d
In semisynthetic diet:				
Coconut oil	20	3	1.9	e
Butter fat	8	4	1.3	f
Butter fat	24	7	2.7	g
Hydrog. peanut oil	20	9	1.0	h

[a] Hirsch and Nailor (1955)
[b] Kritchevsky et al. (1954)
[c] Steiner and Dayton (1956)
[d] Steiner et al. (1959)
[e] Lambert et al. (1958)
[f] Malmros and Wigand (1959)
[g] Funch et al. (1960)
[h] Funch et al. (1962)

Table 2—Influence of roughage on atherosclerosis in rabbits fed cholesterol-free diets[a]

Roughage	Weight gain (kg)	Plasma cholesterol (mg/dl) ± SEM	Degree of atherosis ± SEM
Wheat straw	0.46	114 ± 12	12.7 ± 3.0
Cellulose	0.49	133 ± 10	20.8 ± 2.9
Cellophane	0.46	216 ± 14	37.5 ± 6.8
Cellophane-peat (14:5)	0.47	141 ± 12	10.7 ± 2.0

[a] After Moore (1967). All diets contained 20% butter fat, fed for 40 wk.

coconut oil and the fiber was cellulose (9.8% in the protein diet, 13.1% in the carbohydrate diet and 25.6% in the fat diets). It is evident from Table 3 that the fecal recovery of one oral dose of [^{14}C]-cholesterol was 56–171% higher in the chow-fed rats and, conversely, recovery from serum and liver was 113–206% lower.

In a second experiment (Kritchevsky et al., 1974b) we prepared isocaloric, isogravic diets in which the fiber was either alfalfa or cellulose. The major (50% of calories) dietary components were dextrose, sucrose, corn oil or casein. The alfalfa-fed groups showed lower weight gain (12–27%) than the comparable cellulose-fed rats but serum and liver cholesterol levels were not much different. Absorption of an oral dose of [^{14}C]-cholesterol was considerably lower, however, in each of the alfalfa-fed groups. Fecal excretion of [^{14}C] was 55–83% higher (Table 4). Even 1% of alfalfa added to a semi-purified diet results in significantly reduced absorption of a single dose of radioactive cholesterol (Table 5). Cookson et al. (1967) had shown that dietary alfalfa lowered cholesterol levels in rabbits fed this sterol and speculated that the mechanism of action involved inhibition of absorption. Cookson and Fedoroff (1968) later showed a quantitative relationship between the amount of alfalfa needed to inhibit cholesteremia in rabbits and the dosage of cholesterol.

Table 3—Influence of various diets on cholesterol absorption in rats[a]

Diet	Recovery of [^{14}C] (cpm) ± SEM[b]		
	Serum × 10^3	Liver × 10^4	Feces × 10^6
50% Dextrose	16.25 ± 1.12 a[c]	63.4 ± 5.5 ad	4.30
50% Casein	13.77 ± 1.12 b	52.4 ± 3.9 b	5.45
50% Corn oil	13.13 ± 1.99 c	56.6 ± 2.1 c	4.29
50% Coconut oil	13.27 ± 2.25 d	37.6 ± 5.9 cd	4.10
Chow	5.49 ± 0.90 abcd	12.4 ± 3.2 abcd	7.29

[a] After Kritchevsky et al. (1973). All diets contain cellulose.
[b] Each rat given single oral dose of [4-^{14}C]-cholesterol (0.5 μCi).
[c] Values bearing same letter are significantly different.

Table 4—Distribution of [^{14}C] in rats fed diets containing cellulose (C) or alfalfa (A)

Group	Serum cholesterol (mg/dl)	Recovery of radioactivity (dpm ± SEM)[a]			
		Serum × 10^3	Liver × 10^4	Fecal neutral × 10^5	Fecal acidic × 10^5
Dextrose-C	146 ± 8	6.83 ± 0.55	2.71 ± 0.35	2.01	0.76
Dextrose-A	134 ± 6	3.39 ± 0.60	1.68 ± 0.35	3.64	0.65
Sucrose-C	162 ± 8	7.99 ± 1.04	1.78 ± 0.23	2.50	0.44
Sucrose-A	165 ± 6	4.07 ± 0.79	1.69 ± 0.15	4.17	0.83
Corn oil-C	158 ± 8	7.74 ± 1.09	2.06 ± 0.10	2.05	0.58
Corn oil-A	152 ± 6	1.74 ± 0.42	1.04 ± 0.05	3.74	0.78
Casein-C	153 ± 5	9.87 ± 1.08	3.12 ± 0.35	1.45	0.66
Casein-A	136 ± 8	7.04 ± 0.94	2.61 ± 0.15	2.49	1.37

[a] Rats fed one dose of 0.5 μCi of [4-^{14}C]-cholesterol

Table 5—Influence of 1% alfalfa in semi-purified diets on cholesterol metabolism in rats (3 wk feeding)

	Dietary group (6/group)	
	Semi-purified (SP)	SP + Alfalfa (1%)
Weight gain, g	65 ± 6[a]	62 ± 2
Liver weight, g	7.9 ± 0.4	9.8 ± 0.4*
Cholesterol		
Serum, mg/dl	102.7 ± 5.4	84.3 ± 6.0**
Liver, mg/100g	292 ± 20	213 ± 18
Serum plus Liver Pool, mg	31.3 ± 0.5	27.5 ± 1.4**
Absorption (after 0.5 μCi of [4-^{14}C]-cholesterol)		
Serum (dpm × 10^4)	2.69 ± 0.44	1.66 ± 0.28
Liver (dpm × 10^4)	6.03 ± 0.88	5.97 ± 0.66
Feces (dpm × 10^5)		
Neutral steroid	4.38	6.86
Acidic steroid	1.46	2.00
% Absorption	46.9	19.5

[a] Standard error
* $p < 0.001$
** $p < 0.05$

It would appear, then, that some component(s) in dietary fiber affect cholesterol absorption. Vijayagopalan and Kurup (1970) fed rats diets containing 56% of carbohydrate in which the carbohydrate component ranged from glucose to tapioca starch-bran (5:2). The type of starch and the presence of bran had a significant effect on serum, liver and aorta cholesterol levels. Thus tapioca starch alone gave serum (mg/dl), liver (mg/g) and aorta (mg/g) cholesterol levels of 205, 24.3 and 8.1 whereas tapioca/bran-fed rats exhibited levels of 130, 16.1 and 3.9, respectively. In contrast, a group of rats fed rice starch had cholesterol levels of 348 mg/dl in the serum, 30.7 mg/g in the liver and 13.8 mg/g in the aorta. More recently Vijayagopalan et al. (1973) have reviewed these data and augmented them with results from experiments in which other types of starch were fed (Table 6). A crude correlation between fiber content and cholesterol level can be adduced. Trowell (1972a) has constructed an almost linear correlation between serum cholesterol levels and fiber intake in Americans eating normal or vegetarian diets, using data of Hardinge and his coworkers (1954, 1962). In man, rolled oats (de Groot et al., 1963) and Bengal gram (Mathur et al., 1968) have a significantly hypocholesteremic effect. Diets containing the same fat but different amounts of fiber result in large differences in weight of feces. Antonis and Bersohn (1962) found that a high-fiber, (15g)-butter diet resulted in a threefold increase in stool weight when compared with a low fiber (4g)-butter diet. When the dietary lipid was sunflower seed oil the increase in stool weight on the high fiber diet was only slightly lower (2.7-fold).

Mode of fiber action

The possibility that one mode of action of fiber may be that of the binding of bile salts has prompted experiments in which the binding of sodium tauro- or glycocholate by different foodstuffs was tested (Kritchevsky and Story, 1974; Story and Kritchevsky, 1974). As can be

Table 6—Influence of starches on cholesterol levels in rats[a]

Starch	Fiber content (%)	Cholesterol levels: Serum (mg/dl)	Liver (mg/g)	Aorta (mg/g)
Rice	0.10	348	30.7	13.8
Wheat	0.12	358	41.1	16.0
Corn	0.275	233	26.7	12.4
Bajra	0.436	226	24.9	10.8
Jowar	0.85	142	19.9	8.0
Tapioca	1.25	205	24.3	8.1
Ragi	2.16	93	15.8	5.2
Blackgram polysaccharide	7.1	75	4.6	2.8
Sucrose	—	372	48.2	17.5

[a] After Vijayagopalan et al. (1973). Diets contain 56% carbohydrate, 15% hydrog. peanut oil, 2% cholesterol.

Table 7—In vitro binding of sodium taurocholate by various substances

Substance	% Bound ± SEM: Experiment 1[a]	Experiment 2[b]
Cholestyramine	81.5 ± 0.2	—
Colestipol	57.0 ± 0.5	—
Cellulose	0.5 ± 0.5	—
Cellophane	0.4 ± 0.7	—
Alfalfa	16.9 ± 0.7	34.5 ± 0.2
Wheat straw	1.8 ± 0.8	—
Bran	0.7 ± 0.7	—
Oregano	—	46.3 ± 0.2
Parsley	—	40.8 ± 0.6
Sage	—	24.7 ± 1.0
Celery	—	12.7 ± 0.2
Bagasse	—	5.6 ± 0.4

[a] 40 mg of binding substance, 100 μmoles of sodium taurocholate
[b] 100 mg of binding substance, 100 μmoles of sodium taurocholate

seen from Table 7, alfalfa binds about 20% as much bile salt as does cholestyramine and about 30% as much as colestipol. Cholestyramine and colestipol are pharmaceutical products prepared specifically to bind bile acids and bile salts.

Effect of dietary fiber

We have carried out several experiments which have provided some clues to an effect of dietary fiber. In one experiment (Kritchevsky et al., 1974a) baboons were fed diets containing 40% carbohydrate (glucose, fructose, sucrose or starch), 14% hydrogenated coconut oil and 15% cellulose for 12 months and their blood and tissue chemistries compared with those of baboons maintained on a diet of bread, bananas, oranges, carrots and yams. The baboons on the test diets exhibited aortic sudanophilia and hyperlipidemia. All the animals were given an intravenous dose of [5-^{3}H]-mevalonic acid 2 days before the termination of the experiment. Analysis of the bile lipid radioactivity indicated that the test animals had continued to synthesize cholesterol but had converted much less precursor to bile acids.

Primary/secondary bile acid ratios

Calculation of the ratio of primary (cholic and chenodeoxycholic) to secondary (deoxylic and lithocholic) bile acids shows a lower ratio in the test animals than in the controls. These results, which are summarized in Table 8, suggest that on the semisynthetic diet cholesterol is still synthesized but less is converted to bile acids and the excess is presumably spilled into the serum. From data obtained in an experiment done in rabbits fed a lithogenic diet Kyd and Bouchier (1972) reached a similar conclusion.

We carried out an analogous experiment in rabbits with similar results (Table 9). Chow-fed rabbits excreted four times as much feces as did those fed a semi-purified diet like that fed the baboons.

Biliary cholesterol-specific activity was higher in the group fed the test diet but cholanoic acid-specific activities and primary/secondary bile acid ratios were significantly lower. The data suggest that one reason for the cholesteremic effect of the semi-purified diet is a reduced level of conversion of endogenous cholesterol to bile acids. A hypothetical mechanism of action is depicted in Figure 1.

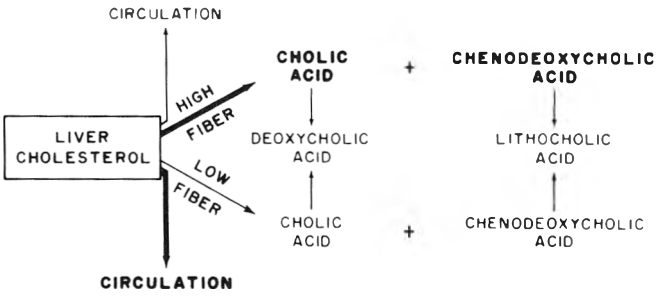

Fig. 1—Hypothetical explanation of effects of diets low or high in fiber on disposition of lever cholesterol. Heavy lines or letters indicate high concentrations.

Table 8—Influence of various diets on baboon serum and bile lipids[a] (fed for 12 months)

Dietary carbohydrate (40%)	Serum, mg/dl ± SEM: Cholesterol	Triglyceride	Bile[b]: P/S[c]	[^{3}H] dpm × 10^3: Sterol	Bile acids	Avg sudanophilia
Fructose	162 ± 10	129 ± 11	0.56	88	3	11.2
Sucrose	152 ± 9	116 ± 8	0.61	30	3	6.7
Starch	156 ± 8	108 ± 5	1.33	47	2	9.3
Glucose	151 ± 11	105 ± 7	0.81	47	2	6.2
Control	113 ± 3	78 ± 4	1.67	59	15	0.02

[a] After Kritchevsky et al. (1974a). Diet also contained 14% hydrogenated coconut oil and 15% cellulose.
[b] Baboons given intravenous dose of 20 μCi/kg of [5-^{3}H]-mevalonic acid.
[c] P/S = primary (cholic, chenodeoxycholic)/secondary (deoxycholic, lithocholic) bile acids.

Table 9—Influence of chow and semisynthetic (SS) diet (6 months) on serum and bile lipids in rabbits[a]

	Group	
	Chow	SS
Serum lipids, mg/dl ± SEM		
Cholesterol	93.0 ± 5.4	2.48 ± 17
Triglycerides	75.8 ±24	130.7 ± 24
Liver lipids, mg/100g ± SEM		
Cholesterol	144 ± 4	225 ± 10
Triglycerides	49 ± 11	122 ± 15
Bile lipids mg/ml ± SEM		
Cholesterol	4.9 ± 1.3	10.8 ± 1.9
Deoxycholic acid	4.72 ± 2.92	13.30 ± 1.82
Cholic acid	0.74 ± 0.23	1.02 ± 0.22
Cholic/deoxycholic	0.16 ± 0.26	0.08 ± 0.02
Avg atheromata	0.0	1.35

[a] SS diet contained: 40% carbohydrate (sucrose/starch); 25% casein; 14% hydrogenated coconut oil; 15% cellulose.

Our findings regarding the bile acids reflect those of Portman (1960) who found that in rats fed chow, the half-life of cholic acid was half that of rats fed sucrose, the cholate pool doubled and the cholic acid excreted was five times higher. Addition of 20% fiber to the sucrose diet reduced the half-life of cholic acid and increased cholate excretion. Portman concluded that his results were due to modification of bile acids during digestion as well as to rates of reabsorption.

CONCLUSIONS

NOT ENOUGH is known about fiber composition or fiber action to attribute to it any single mode of action. Wheat straw exerts some effects on lipids (Moore, 1967) that cannot be due simply to binding of bile salts (Kritchevsky and Story, 1974). The factors of transit time (Walker et al., 1970; Holmgren and Mynors, 1972), influence on intestinal flora and effects upon metabolites other than bile salts must be considered. Present data suggest, however, that dietary fiber can no longer be regarded as an inert filler. Nutritionists, food technologists and medical scientists must regard fiber as an integral dietary ingredient whose role may be as important as those of protein, fat, carbohydrate, vitamins or minerals.

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SYMPOSIUM: Nutritional Perspectives and Atherosclerosis
LIPIDS IN ATHEROSCLEROSIS

THE ROLE that individual foods play in the nutritional perspective, that is, their actual role, in atherosclerosis is still not clear. Recommendations are being made and conclusions drawn without knowledge of all the factors involved. For example, the final report of the National Diet Heart Study (1968) stated "Extensive evidences implicate diet as a key factor in the etiology of atherosclerosis and suggests that the disease can be prevented by changes in diet, particularly by lowering serum cholesterol level." In addition, a recent policy statement of the American Medical Association Council on Food and Nutrition (1972) and the Food and Nutrition Board of the National Academy of Sciences indicated how changes in diet could be accomplished: "Generally such lowering can be achieved most practically by partial replacement of the dietary sources of saturated fat with sources of unsaturated fat, especially those rich in polyunsaturated fatty acids." This joint statement concluded: "There is abundant evidence that the risk of developing CHD [cardiovascular heart disease] is positively correlated with the cholesterol in the plasma." Further research indicates that these statements do not reflect the full picture. Such a nutritional perspective has not fully considered that dietary sources of saturated fat and cholesterol, that is, meat, eggs and dairy products, serve as the major source of protein, vitamins and minerals in the American diet (Connor et al., 1968). It has been assumed that less cholesterol is deposited in the arteries when the serum cholesterol level is lowered by the addition of polyunsaturated fats to the diet, an assumption that cannot be tested in human subjects.

POSSIBLE ROLE OF *LIPIDS* IN DEVELOPMENT OF ATHEROSCLEROSIS

THE ASSUMPTION that a high serum cholesterol level accelerates the development of atherosclerosis has been found valid in many studies on experimental animals. The high serum cholesterol levels can be obtained by feeding animals high fat diets which contain 1–2% crystalline cholesterol, or a cholesterol intake equivalent in man to the consumption of 30–60 eggs/day. However, this assumption does not explain the development of CHD in subjects with serum cholesterol levels below 250 mg %. Another explanation for the development of CHD in these subjects may possibly be found in the character of the dietary fats we consume as well as other yet unknown factors.

It has been shown that the endothelium layer of the abdominal aorta can be ruptured mechanically by inserting an inflatable balloon into the upper femoral artery and pushing it upward into the aorta. Lipids infiltrate the smooth muscle cells adjacent to the ruptured endothelium layer of cells to produce changes similar to those noted in the development of atherosclerosis (Nam et al., 1973). Furthermore, Dr. H. Imai, of the Albany Medical School, who is working with us on our studies on swine atherosclerosis, has found dead cells within the layer of smooth muscle cells in the intima and media of newly born swine (personal communication).

Immunological evidence indicates that the very low density lipoprotein fraction of the serum furnishes the lipids that accumulate in the intima layer of the aorta. However, studies with radioactively labeled components have shown that both lipid infiltration and lipid synthesis in situ can occur in isolated arterial tissue. The incorporation of ^{32}P-phosphate and ^{14}C-choline into normal and atherosclerotic rabbit aortas has been studied in vitro (Newman et al., 1966). The same results as observed in vivo were found, namely an enhanced synthesis of phospholipids by the atheromatous aorta, localized primarily in the intima. The difference in distribution of ^{32}P between the two most active phospholipid classes, phosphatidylcholine and phosphatidylinositol, of normal and atherosclerotic aortas was similar in vivo and in vitro. In the normal aorta, phosphatidylinositol had a higher percentage of ^{32}P than phosphatidylcholine; in the atheromatous aorta, these percentages were reversed. The studies in vitro were used as evidence to show that the enchanced synthesis observed in vivo was probably not mediated by neural factors or stretch of the aorta, and that synthesis in situ could account for the higher amounts of phospholipid present in the atheromatous aorta.

It has been shown by Van Deenen and co-workers (Van Den Bosch et al., 1968; 1969) that the replacement of an unsaturated by a saturated fatty acid influences the physical characteristics of the phospholipid into which it is incorporated and maintains the physical properties of the phospholipid molecule between certain limits. Data on liquid crystals and synthetic membranes support the hypothesis that the properties of membranes are dependent on the physical characteristics of the fatty acid composition of the phospholipids (Jones et al., 1969; Pande et al., 1968; Demel et al., 1972). Van Deenen and co-workers (DeKruyff et al., 1973) recently incorporated elaidic instead of oleic acid into the phospholipids of *A. laidlawii*. They noted a difference in the energy contents of the phase transitions of the isolated lipids which they believed may have significance to the liquid crystalline state as cholesterol was shown to preferentially interact with lipids which are in the liquid crystalline state. As phospholipids are important components in the cell membranes that make up the myocardium and the intima, the fatty acid composition of these phospholipids may be important to the rate at which lipid infiltration can occur through the cell membrane. It seems important to study their role in the step by step biochemical changes in the subcellular fractions of involved and uninvolved aortic tissue. Furthermore, these changes should be correlated with the biophysical properties of cell membranes and with the changes in pathology that have been noted at the cellular level. Such a correlation has been difficult to date as a visual inspection of an aorta cannot differentiate between the involved lipid-ladened areas and the noninvolved or lipid-free areas during the initial stages in the development of atherosclerosis. The presently accepted method of staining of the aorta with Sudan IV extracts a portion of the lipid and inactivates the enzymes in the aorta. We have found that the spraying of the freshly excised aorta with a solution of Rhodamine G and visualization under ultraviolet light provide for a good means of differentiation between the involved

and noninvolved areas (Cho and Kummerow, 1974).

POSSIBLE ROLE OF THE FOOD INDUSTRY IN THE NUTRITIONAL PERSPECTIVE IN ATHEROSCLEROSIS

THE FOOD INDUSTRY has the technology to provide new food items which may reduce the risk of developing CHD. However, to do so will require information in at least four areas:

(1) The level of total visible fat intake that will minimize the nutritional perspective of dietary fat in atherosclerosis;
(2) The ideal percentage of PUFA in such dietary fats;
(3) The percentage of PUFA than can be present in the trans form in shortening, margarine and salad oils; and
(4) The possible role of cholesterol-containing food items in the development of atherosclerosis.

Level of total visible fat intake that will minimize the nutritional perspective of dietary fat in atherosclerosis

Both the American Medical Association and the National Research Council have recommended that the percentage level of fat intake should be reduced. However, such a recommendation may not be nutritionally sound as it may only increase the consumption of carbohydrate. The extra calories from carbohydrates are readily converted to saturated fatty acids or the unsaturated ω9 series of fatty acids which do not have the same biological function as the ω6 series of polyunsaturated fatty acid. As fat calories have more satiety value and are less expensive than an equivalent amount of calories from carbohydrates, it would be more economical to stay at our present percentage level of total fat and decrease the number of calories while maintaining a "well balanced" diet. However, if the level of protein intake is reduced, the level of intake of high fat food items should be reduced.

The classical nutrition studies at INCAP has shown that fat calories prevent a biological "waste" of the more expensive protein calories. In the development of substitute protein food items, it would be desirable to add enough fat calories to prevent their deamination and assure their full utilization.

Ideal percentage of PUFA in food items

The human diet contains a mixture of dietary fats that presumably supply an adequate amount of linoleic acid, yet human adrenal cholesterol esters contain the elongated (n-9) series of fatty acids which may indicate the presence of less than an optimum level of dietary (n-6) fatty acids (Table 1). The percentage of total (n-9) polyunsaturated C_{18} and C_{20} fatty acids in the human adrenal (Raggatt et al., 1972) was in the same approximate range as found in rats fed 20% of a PUFA-free hydrogenated soybean oil and 2% corn oil or 8 and 14%, respectively. The adrenal cholesterol esters from rats fed only corn oil contained 3%, while those fed only hydrogenated soybean oil contained 34% of the (n-9) fatty acids. The total (n-6) fatty acids of human adrenal lipids was comparable in amount to that found in rats fed 2% corn oil. The hearts from rats fed 20% hydrogenated soybean oil contained significantly more total lipid than the hearts from rats (Egwim and Kummerow, 1972a) fed 20% beef tallow. An analysis of the lipids extracted from the adrenal glands, liver, heart and the erythrocytes of rats fed an adequate amount of linoleic acid (C18:2ω6) has indicated that these tissue contain a substantial amount of elongated C_{20} and C_{22} PUFA fatty acids which retained the (n-6) configuration. Diets rich in linoleate, corn oil, or nutritionally adequate with respect to linoleic acid, milk fat or beef tallow, lead to an elevated level of (n-6) long chain unsaturated fatty acids in the cholesteryl esters and phospholipids in adrenal, heart and liver tissue (Egwim and Kummerow, 1972b) (Table 2). The presence of the 5% of vaccenic acid (t-18:1) (n-11) in butterfat did not interfere with the accumulation of C_{20} and C_{22} PUFA acids. The percentage of PUFA in butterfat or beef *tallow could* be increased by the addition of corn or cottonseed oil. However, an increase in PUFA could best be obtained by increasing the PUFA content of shortenings and margarines.

Table 1—Composition of adrenal cholesterol esters[a]

Obtained from rats fed:	Total polyunsaturated fatty acids: C_{18+20} % Amount ω6	ω9
20% Hydrogenated fat	3.4	34.2
20% Hydrogenated fat + 2% corn oil	9.1	14.2
20% Corn oil	29.5	3.0
Human[b]	24.9	8.3
Human[c]	7.0	4.9

[a] J. Nutr. 101: 315 (1971)
[b] Lipids 5: 743 (1970)
[c] Lipids 7: 474 (1972)

Table 2—Concentration of total ω9 and ω6 fatty acids (C_{20} and greater) of heart phospholipids[a]

Diets	15 wk ω9	15 wk ω6	20 wk ω9	20 wk ω6
Corn oil	–	30.3	–	23.6
Milk fat	–	3.1	–	32.0
Beef tallow	1.5	5.2	4.9	31.6
Hydrog. fat	5.2	4.6	3.3	17.8
Fat-free	20.7	–	22.4	12.8

[a] Results are summation of total ω9 and ω6 fatty acids, respectively, expressed as percent of total fatty acids [J. Lipid Res. 13 500 (1972)].

Percentage of PUFA that can be present in the trans form in shortenings, margarines and salad oils

Americans consumed approximately ten billion pounds of "visible" fats per year. The major source of fat has been listed as 6.3 billion pounds of soybean oil which was converted to baking or frying fats, salad oils or margarines. The stabilization of soybean oil towards autoxidation (rancidity) by means of hydrogenation has made it possible to supply an abundance of economical and sensory acceptable calories to the American diet. In commercial practice, the undesirable linolenic acid in the soybean oil is converted to the more stable monoenoic (oleic) and saturated fatty acids through hydrogenation of the double bonds. In this process, the double bonds are also isomerized and as much as 50–60% of them are converted from the natural cis to trans forms. Stick margarine contains from 25–35%, tub margarines 15–25%, shortenings 20–30% and salad oils contain from 0–15% trans fatty acids. Household Consumption Data (USDA, 1971) indicate that margarine represents 7%, shortenings 13.2% and cooking and salad oils 12.4% of the visible fat intake. On this basis, the total trans fatty acid intake from visible fat is approximately 8%.

Does the 8% of total trans fatty acids in the diet have any nutritional or biological consequences? Such a question may be considered from both a structural and a functional parameter. The structure parameter is complicated by the interplay between the trans fatty acids and the es-

Table 3—Summary data on ten groups of swine fed the basal diet plus various fat and cholesterol supplements

Diet	Total serum lipid mg%	Serum cholesterol mg%	RBC L/O	Intima cholesterol mg/g	Atherosclerosis[b] %	Lesions[c]
Basal	273 ± 12	95 ± 5	0.9	8.6	6.0	3(10)
+20% Beef tallow	331 ± 13	124 ± 5	0.6	8.0	5.2	1(11)
+20% Rearranged fat	342 ± 19	125 ± 8	2.3	8.9	3.8	0(11)
+20% Corn oil	276 ± 21	104 ± 7	2.4	9.0	5.0	2(12)
+10% Used fat and sugar	362 ± 26	131 ± 11	0.8	9.6	8.6	3(12)
+20% trans fat	338 ± 20	138 ± 9	0.7	10.4	10.0	7(12)
+20% Butterfat	332 ± 15	120 ± 7	1.0	7.2	7.3	2(9)
+Whole egg powder	303 ± 14	112 ± 5	0.8	7.7	4.8	1(11)
+Egg yolk powder	286 ± 13	98 ± 5	1.0	7.2	4.2	0(12)
+Crystalline cholesterol	245 ± 19	93 ± 3	1.3	9.1	5.2	2(12)

[a] Basal—1,745 lb ground yellow corn, 200 lb soybean meal, 55 lb lysine supplement, vitamin-mineral premix, egg powder fed at cholesterol equivalent of 500 mg/day/200 lb animal weight.
[b] Atherosclerosis—% of total area of aorta
[c] Lesions—number raised plaques.

sential ω6 series of PUFA. We are indebted to Klenk (1965), Stoffel and Ahrens (1959), Lands et al. (1966), Privett and Blank (1964), De Tomas et al. (1963), Mohrhauer and Holman (1963), Sgoutas (1968, 1970) and Sgoutas and Kummerow (1969, 1970) for unraveling this interplay. Briefly, these workers have shown that in the absence of dietary trans fatty acids the ω6 series of PUFA esterify the β position of phosphatidylcholine and that in the absence of dietary sources of the ω6 series of fatty acids, the elongated ω9 series of fatty acids esterify this position. The trans fatty acids esterify the α position in the presence of dietary ω6 PUFA. However, in the absence of dietary ω6 PUFA, the elongated trans fatty acid esterifies the β position. The PUFA in the β position of phosphatidylcholine esterfies cholesterol. The interplay between the esterification and hydrolysis of the cholesterol esters is very precise. The elongated ω6 PUFA preferentially esterifies cholesterol and is also preferentially hydrolyzed (Goller et al., 1970). However, in the absence of elongated ω6 PUFA, the ω9 series of fatty acids esterify cholesterol. In the presence of trans fatty acids, the ω6 PUFA preferentially esterifies cholesterol; however, the trans fatty acids also esterify cholesterol. Once esterified, ω6 cholesterol esters are preferentially hydrolyzed which can result in the accumulation of cholesterol esters of the elongated ω9 fatty acids in the tissue.

In a recent study in our laboratory (Mizuguchi et al., 1974), 6-month old swine were fed a commercially available hydrogenated fat which contained less than 1% C18:2 and 40% trans fatty acids in its mixed fatty acid composition (Table 3). The 6-month old swine were fed for 8 months 10 or 20% of the hydrogenated fat in addition to a diet which contained all of the essential minerals and vitamins; this basal diet furnished the equivalent of 14.3% protein and 3% corn oil. Other groups of swine were fed tallow, corn oil, used fat, butterfat, egg yolk, whole egg powder or crystalline cholesterol equivalent to 2 eggs/200 lb animal weight.

The results indicated that the total lipids in the hearts of swine fed hydrogenated fat contained approximately 9% elaidate after 8 months on the diet. The plasma lipids contained approximately 5.6% elaidate in the cholesterol esters, 4.3% in the triglyceride and 12.3% in the phospholipid fraction, respectively. As would be expected, the LDL and HDL fraction of plasma lipoproteins from swine fed hydrogenated fat contained more lipid than those on the basal diet.

The total plasma lipid level was higher in swine fed the "trans" fat than in those fed tallow, non-trans hydrogenated fat or corn oil and the plasma cholesterol level was higher than the cholesterol level in plasma from swine fed diets which contained cholesterol. The serum cholesterol level was also elevated in the swine fed beef tallow or butterfat. However, it was not elevated in swine fed egg yolk, whole egg or crystalline cholesterol at a level equivalent to 2 eggs/day/200 lb of animal weight. Supplementation with corn oil resulted in lower serum cholesterol levels than supplementation with beef tallow or butterfat. However, supplementation with rearranged fat stimulated approximately the same response as corn oil. The rearranged fat was prepared from completely hydrogenated soybean oil and cottonseed oil.

In agreement with the conclusions of the National Diet Heart Study (1968), the present results indicated that the L/O ratio of the lipids in the erythrocytes was higher when corn oil rather than when "hard" fats such as butterfat or tallow served as a dietary fat. Furthermore, the C20:4ω6 level of the erythrocytes from those fed trans fat was lower than in swine fed the other dietary fats. Although the presence of trans fatty acids in the dietary fat decreased the amount of linoleic acid in the lipids of the erythrocytes, the L/O ratio of these lipids was higher than the L/O ratio of the lipids obtained from human erythrocytes in the Nation Heart Study. The L/O ratio has been used as an index of adherence to a high PUFA diet. The present study indicated that a high L/O ratio dietary fat will lower serum cholesterol levels and it has been assumed that the L/O ratio of a dietary fat influenced the degree of atherosclerosis (National Diet Heart Study, 1968). However, such an assumption cannot be proven with human experimental subjects. Furthermore, these parameters were of no value in predicting whether raised plaques or fatty streaks had developed in the arteries.

The normal intima obtained from all ten groups showed a similar concentration of total lipid and lipid classes. The total lipid content of the involved intima was increased about 70% or as much as 42.5 mg/g tissue. The highest concentration of cholesterol was noted in the swine fed trans fat or 10.4 mg/g tissue. However, the involved intima from all ten groups did not show statistically significant differences in concentration of total lipid, cholesterol and other lipids. Both the normal and the involved intima did not show significant differences when compared to the proportion of each lipid class to the total lipid.

Fatty streaks were observed in all thoracic and abdominal aorta, whereas raised lesions were observed around the

terminal portion of the abdominal aorta and the orifices of celiac and renal arteries. The swine fed trans fat had the largest amount of atherosclerosis or 10% of involvement. The aorta of 7 out of 12 swine fed trans fat had raised lesions (58.3%), whereas raised lesions were observed in only 14 out of 100 swine fed the other diets (14%). The aortas from those fed the 10% used fat and sugar had the second largest amount or 8.6% of involvement.

The high calorie to protein ratio of the dietary fat as well as other yet unknown dietary variables may have contributed to the susceptibility of these swine to the development of atherosclerosis. Furthermore, the presence of a high percentage of trans fatty acids in a dietary fat may require the presence of more than 2–3% linoleic acid to preserve the integrity of the ω6 phospholipids in the cell membrane of the smooth muscle cells in the intima. The hydrogenated fat that was used in this study contained less than 1% linoleic acid and between 40–50% trans fatty acids. In two separate studies, Zalewski and Kummerow (1968) and Carpenter and Slover (1973), commercial margarines were found to contain from 3–43% linoleic acid and from 15–54% trans fatty acids.

The level of dietary linoleic acid in a fat which contains "elaidinized" fat, that is, trans fatty acids may be important to atherogenesis. It was shown by McMillan and co-workers (1963) that a higher serum cholesterol level and more atherosclerosis occurred in rabbits fed elaidinized olive oil than in those fed olive oil. However, such differences were not found significant in rabbits fed elaidinized linoleic acid which contained 18% cis-cis linoleic acid (Weigensberg and McMillan, 1964). More PUFA may be necessary when consumed with sources of trans fatty acids. In fact, previous studies have shown that a greater percentage of trans fatty acids are incorporated into tissue in the presence than in the absence of linoleic acid (Johnston et al., 1958). The inclusion of 2% dietary linoleic acid in a high trans hydrogenated fat diet facilitated incorporation of elaidic acid in adipose tissue, triglycerides, cholesterol esters and phospholipid fractions. The increases were approximately 10% in adipose tissue, 4% for triglycerides, 5% for cholesterol esters and variable in phospholipids (Sgoutas et al., 1973).

The percentage of elaidinized or trans fatty acids in a culinary fat seemed to determine the degree of response in human subjects. When fed as 40% of total calories, a hydrogenated fat which contained 35% trans fatty acids increased serum cholesterol level (Vergroesen, 1972) but one which contained 10% of trans fatty acids had no elevating effect on serum cholesterol levels (Erickson et al., 1964). The triglycerides, cholesterol esters and phospholipids in human serum contained approximately the same level of trans fatty acids as 6 months old swine which had been fed for 8 months a hydrogenated fat which contained 40% trans fatty acids (Kummerow et al., 1973) (Table 4). The red blood cells from these subjects contained from 0 to 5.3% trans fatty acids and had an L/O ratio of 0.4 to 1.0. Human tissue contains trans fatty acids (Johnston et al., 1958): approximately 2.4–12.2% in adipose tissue, 4–14% in liver, 4.6–9.3% in heart and 2.3–8.8% in the aorta.

Table 4–Concentration of elaidate in lipid classes of human as compared with swine plasma

	Swine[a]	Human
Triglycerides	4.3%	2.1–6.7%
Phospholipids	12.3%	3.1–5.7%
Cholesterol Esters	5.6%	1.7–5.1%

[a] Fed 20% hydrogenated fat (40–50% trans) for 8 months

It is possible that the percentage of cis and trans isomers as well as the amount of PUFA in a culinary fat may be important to its possible role in the development of atherosclerosis. This role cannot be studied in man as effectively as in swine as an animal model. I believe that such a study should be carried out with commercially available hydrogenated fats containing various levels of cis-cis linoleic acid. The results to date indicate that a trans-free hydrogenated fat may maximize the biological worth of the ω6 series of PUFA (Lands et al., 1966; Privett and Blank, 1964; Lands, 1965).

Possible role of cholesterol-containing food items in the development of atherosclerosis

To date, the choice of food items which contain protein has been governed by consumer preference for animal protein sources and by individual taste. As animal proteins become more expensive and less are consumed, the biological worth of substitute protein sources becomes increasingly more important and food intake governed solely by taste will become more critical. It may have been possible to indulge in the consumption of empty calories (soft drinks and potato chips) on the present high level of animal protein intake. However, if this level of animal protein intake is reduced to a crucial level, two developments seem necessary: (1) to make available substitute food items equivalent to animal protein in nutritional value; and (2) either to remove the source of empty calories from the diet or to enhance their food value by the addition of essential nutrients. The food industry can increase its efforts to reach these goals so that we will not be caught in a nutritional crisis which will have much more serious consequences to our health and well being than the energy crisis. To date, the complete protein that is produced by beef cattle and dairy cows from marginal grasslands has been more economical to produce than substitute proteins. Animal protein will continue to serve as a substantial source of protein as only 30% of our agricultural lands yield crops which can be processed into foods for direct human consumption (USDA, 1973). If the practice of "finishing" 700-lb grassland beef with corn is abandoned, the amount of beef available at the supermarket will be cut to half of what is presently available. Furthermore, as grass-fed beef it too "tough" to use as roasts and steaks, it will have to be consumed as ground meat. A study on the ideal balance between direct and indirect consumption of cereal grains, therefore, seems necessary in order to use our grassland at maximum efficiency (Hegsted, 1974).

The development of cholesterol-free protein sources cannot be based on food composition data alone; it must be based on nutritional value as biological need is more important than technological expendiency. In its attempt to comply with the recommendations of the American Medical Association and the National Research Council, segments of the food industry has provided a nutritionally inferior product. The nutritionally inferior product is in the form of a cholesterol-free substitute for shell eggs.

The cholesterol-free egg substitute contains, according to the label on the carton, "egg white, corn oil, nonfat dry milk, emulsifiers (vegetable lecithin, mono and diglycerides and propylene glycol monostearate), cellulose and xanthan gums, trisodium and triethyl citrate, artificial flavor, aluminum sulfate, iron phosphate, artificial color, thiamin, riboflavin and vitamin D" (Table 5). A comparison of the nutrients in 100g of Egg Beaters on Second Nature egg substitute with the nutrients in 100g of "farm fresh eggs" indicates a list of nutrients which should be able to meet the growth requirements of weanling rats (Navidi and Kummerow, 1974). However, the pups from the mothers fed Egg Beaters averaged 31.6g and those fed whole egg averaged 66.5g in weight at 3 wk of age as compared to 70g for pups from those fed Purina Laboratory Chow. Both the mothers and pups fed Egg Beaters developed diarrhea within 1 wk; those fed whole egg did not develop diarrhea. The pups fed the egg mixtures were weaned at 5 wk of age. All of those fed Egg Beaters died within 3–4 wk after weaning. The general appearance of the rats fed Egg Beaters indicated a gross deficiency in one or

Table 5—Comparison of nutrients in Egg Beaters or shell eggs with nutrient requirements of growing rats

Nutrients	100g of Egg Beater	100g of Egg	Requirement of growing Rat—% Diet[a]
Protein	11g	12.8g	13.3g
Fat	12.5g	11.4g	5.5g
Calories	166.66	160	GE/day 76 for each rat (444 Kcal/100g).
Ca	81.6 mg	54 mg	560 mg
P	71 mg	204 mg	440 mg
Na	181 mg	122 mg	60 mg
K	213 mg	128 mg	200 mg
Iron	1.8 mg	2.2 mg	38.9 mg
Cholesterol	<1.6 mg	550 mg	—
Vitamin A	1350 I.U.	1180 I.U.	0.67 mg (retinol/kg)
Vitamin D	43 I.U.	50 I.U.	111.1 (I.U./Kg)
Thiamine	0.13 mg	0.1 mg	0.14 mg
Riboflavin	0.43 mg	0.3 mg	0.28 mg
Choline chloride	b	582 mg	83.3 mg/100g
Ca pantothenate	b	2.7 mg	0.89 mg/100g
Vitamin B_6	b	0.3 mg	0.78 mg/100g
Vitamin B_{12}	b	0.001 mg	0.00056 mg/100g
Biotin	b	0.04 mg	0.1 mg/100g

[a] "Nutrient Requirements of Laboratory Animals." No. 10, 2nd ed., 1972. National Academy of Sciences, Washington, D.C.
[b] Not listed

more nutritional factors as compared to those fed whole egg. As the animals had a tendency to become coated with the Egg Beaters, the animals were washed gently with a mild detergent solution and dried with paper towels. The washing removed some of the hair as well as the Egg Beaters.

Neither Egg Beaters nor shell eggs serve as a single food source in the human diet. Furthermore, both Egg Beaters and shell eggs are subjected to heat treatment and are not consumed in the raw state. The rat pups were weaned at 5 instead of the usual 3 wk in order to provide the advantage of rat milk supplementation for them. However, it is evident that shell eggs, which contain the lipotropic-ladened egg yolk, furnish one or more nutritional factors which are absent in Egg Beaters. These nutritional factors are no doubt present in the common food items which comprise the diet of human adults and could probably be added to the Egg Beaters formulation. However, these nutritional factors may not be present in adequate amounts for infants fed *milk and Egg* Beaters instead of egg yolk from a soft boiled egg. The Council statement (AMA, 1972) under point (3) should be considered and "care be taken to assure that the dietary advice given does not compromise the intake of essential nutrients." This statement should also be considered in the quest of food items free of cholesterol in the diets of infants. Furthermore, the assumption that the consumption of eggs, meat and dairy products by growing children should be minimized and replaced with PUFA may result in nutritional disaster (Fredrickson and Levy, 1971). The protein calorie-ratio is important to rapid growth in farm animals and poultry and deserves further study in man in the development of new food items.

SUMMARY

IT SEEMS EVIDENT that dietary advice should take into consideration the availability of food items, their composition and their subtle impact on the nutrition and the biochemistry of the individual cell. The food industry has a vital role to play in furnishing new food items which have been thoroughly tested for more than their freedom from toxic compounds. New food items should be considered in terms of their total nutritional impact as such food items will become increasingly more important to human welfare in a world of rapidly shrinking supplies of food items which have served as the main sources of proteins, vitamins and minerals for countless generations.

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SYMPOSIUM: Nutritional Perspectives and Atherosclerosis
EFFECTS OF DIETARY PROTEIN AND CARBOHYDRATE ON PLASMA CHOLESTEROL LEVELS IN RELATION TO ATHEROSCLEROSIS

THE FIRST clear demonstration that diet plays a role in the development of atherosclerosis was reported by Ignatowski (1908, 1909), who investigated the effects of feeding animal products such as meat, milk and eggs to rabbits and observed that some of the animals developed lesions in the aorta resembling those seen in human atherosclerosis. He concluded that this was due to injurious effects of the animal protein on the arterial wall, but later experiments by Stuckey (1912) and Wesselkin (1913) provided evidence that fatty substances, rather than the protein in these foods, were primarily responsible for the lesions. After Anitschkow and Chalatow (1913) succeeded in producing typical lesions of atherosclerosis in rabbits simply by feeding cholesterol dissolved in vegetable oil, most subsequent workers concentrated on cholesterol feeding as a method of producing experimental atherosclerosis and possible effects of other dietary components was largely ignored, although Meeker and Kesten (1940, 1941) provided clear evidence of the atherogenicity of casein in rabbits.

The studies of Lambert et al. (1958), Wigand (1959) and Malmros and Wigand (1959) showed that hypercholesterolemia and atherosclerosis could be induced in rabbits by feeding purified rations devoid of cholesterol and this has been confirmed in a number of other laboratories (Funch et al., 1960; Gresham and Howard, 1962; Moore and Williams, 1964a, b, c; Kritchevsky and Tepper, 1965; 1968; Wilson et al., 1973). These effects can be obtained with low fat semisynthetic diets and with semisynthetic diets containing saturated fats such as butter and coconut oil, but are largely prevented by the presence of substantial amounts of unsaturated fats in the diets.

Malmros and Wigand (1959) thought that the effects were due to essential fatty acid deficiency, but this does not appear to be a satisfactory explanation. Atherosclerosis does not develop in rabbits maintained on commercial laboratory chow diets (Kritchevsky, 1964) but the protective effect cannot be ascribed to the small amount of unsaturated fat present in such diets. Kritchevsky and Tepper (1968) showed that extracting the lipid from Purina rabbit chow and adding it to a semisynthetic diet did not basically alter the results obtained by these two different types of diet. Such findings indicated that nonlipid components of the diets were probably responsible for the observed differences and some attempts have been made to identify such components. Howard et al. (1965) varied the composition of the basic semisynthetic diet by replacing the vitamin mixture with yeast and cabbage, the salt mixture by bone meal or bone ash, the corn starch by corn meal, the glucose, sucrose and potassium acetate by corn starch and the casein by purified soya protein. They reported that none of these alterations had any effect on incidence or severity of atherosclerosis, but they did find that replacement of the casein by whole soya flour or hexane-extracted soybean meal was effective in preventing the hypercholesterolemia and atherosclerosis in rabbits given semisynthetic diets.

Table 1—Composition of basic semisynthetic cholesterol-free diets[a]

Ingredient	Low fat (%)	High fat (%)
Casein (Vitamin-free)	27.0	30.0
Dextrose	60.0	44.0
Celluflour	5.0	5.0
Salt mix[b]	4.0	5.0
Molasses (75% sol)[c]	2.0	–
Fat	1.0	15.0

[a] The protein content of the high-fat diet was increased somewhat to compensate for the higher caloric value of the fat. The diets also contained supplements of water-soluble and fat-soluble vitamins. In the low-fat diets the fat-soluble vitamins were dissolved in corn oil to provide a source of essential fatty acids.
[b] Formulated according to Phillips and Hart (1935) and supplied by Nutritional Biochemicals, Cleveland, Ohio.
[c] Molasses was added to reduce the dustiness of the diets.

Effects of sugars and starch

Kritchevsky et al. (1968) investigated the effects of various carbohydrates in a semisynthetic cholesterol-free ration containing 14% hydrogenated coconut oil. They reported that starch was the most hypercholesteremic and atherogenic of the carbohydrates used and glucose was the least. Intermediate results were obtained with sucrose and partially hydrolyzed starch. In subsequent experiments (Kritchevsky et al., 1973), fructose was found to be as atherogenic as sucrose and lactose to be slightly less atherogenic than glucose. In these later studies, starch appeared to be less atherogenic than sucrose although still more atherogenic than glucose. The diets were fed for 4, 6 and 10 months respectively, and the severity of atherosclerosis tended to increase with more prolonged feeding. The serum cholesterols were considerably higher at 10 months than after 4 or 6 months on these semisynthetic diets.

Studies on the effects of sucrose, glucose and starch fed to rabbits in fat-free, cholesterol-free semisynthetic diets were reported by Malmros (1969). Sucrose gave the highest serum cholesterol levels and starch the lowest and after a year only the group on sucrose diet showed evidence of atherosclerotic changes. The source of starch was not indicated in any of the above studies.

Experiments in our laboratory (Carroll, 1971) showed that addition of glucose (dextrose) to commercial feed in the ratio of 1:3 had no effect on rabbit plasma cholesterol levels, while addition of casein in similar amounts produced a definite hypercholesterolemia. Earlier studies by Meeker and Kesten (1940, 1941) also indicated that casein produces a hypercholesterolemia in rabbits in the absence of dietary cholesterol and they showed that it was atherogenic as well. On the

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other hand, they found that soybean flour did not give rise to hypercholesterolemia and atheromata and in fact appeared to exert an inhibitory effect on atherosclerosis induced by dietary cholesterol.

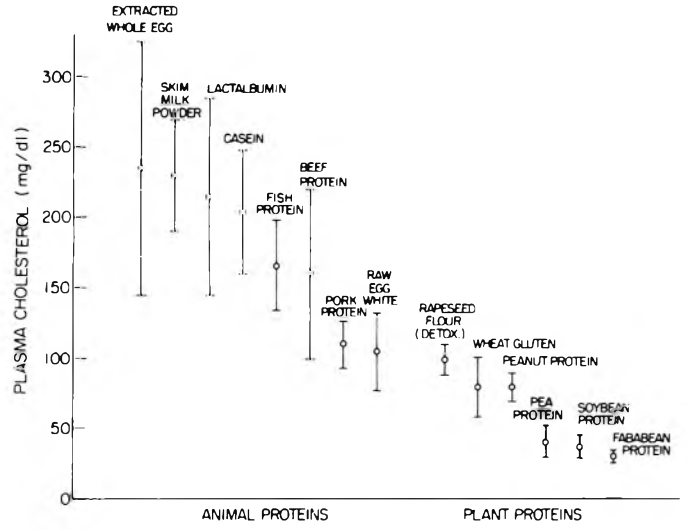

Fig. 1—Effect on total plasma cholesterol in rabbits of low fat, cholesterol-free semisynthetic diets containing different proteins. Diets were fed for 28 days and values shown are the Mean ± S.E.M. for groups of five to six rabbits, except that only four rabbits were fed the extracted whole egg diet. Lipid-free extracts of whole egg powder, beef, pork and peanut protein were prepared in our laboratory. Fish protein concentrate was kindly provided by Dr. L. Regier, Fisheries Research Laboratory, Halifax, N.S.; detoxified rapeseed flour by Dr. J.D. Jones, Food Research Institute, Research Branch, Agriculture Canada, Ottawa, Ont.; pea protein and fababean protein by Dr. C.G. Youngs, Prairie Regional Laboratory, National Research Council, Saskatoon, Sask. The last two preparations contained a small amount (approx 5%) of naturally-occuring lipid. All other protein preparations were obtained from commercial sources.

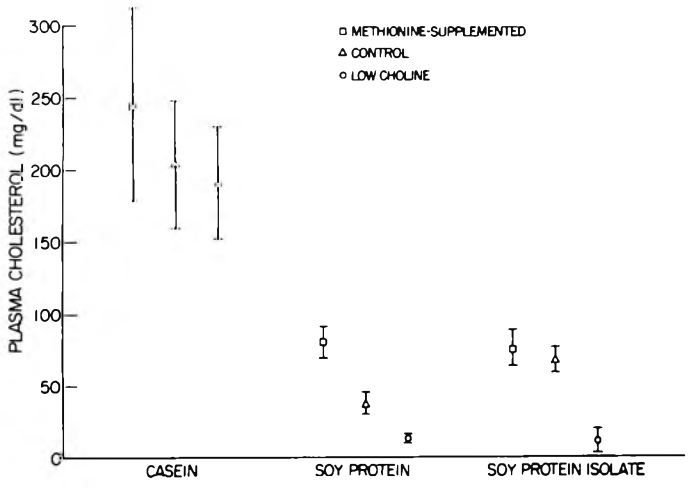

Fig. 2—Effect on total plasma cholesterol in rabbits of diets supplemented with methionine or low in choline and containing casein, soy protein or soy protein isolate as source of protein. Values shown are Mean ± S.E.M. for groups of four to seven rabbits fed for 28 days. DL-Methionine was added at the level of 2% of the dietary protein. Choline was reduced from 150 mg/kg of the diet to 150 μg/kg in the low choline diets. The soy protein isolate (Promine-R) was generously supplied by Dr. E.W. Meyer, Central Soya, Chemurgy Div., Chicago, Ill.

Effects of nonlipid dietary components

More recent experiments in our laboratory (Hamilton, 1972) were designed to investigate systematically the effects of different nonlipid dietary components on the level of plasma cholesterol in rabbits on cholesterol-free diets. The ingredients of the basic diets used for these studies are listed in Table 1. Diets of the low-fat type were used for most experiments and were fed to groups of 4–7 male New Zealand white rabbits for 28 days in each case.

Diets containing animal or plant proteins

Results obtained with diets containing proteins from various sources are shown in Figure 1. Although there was considerable variation, diets containing animal proteins generally gave higher plasma cholesterol levels than diets containing plant proteins. Most of the plant protein preparations contained only about 60% protein and since the diets were formulated to be isonitrogenous, this means that substantial amounts of nonprotein material from such sources were added as well. However, in the case of soybean protein, an isolate containing about 95% protein gave results similar to those obtained with the crude preparation (Fig. 2). Figure 2 also shows that the difference between results obtained with casein and soybean protein was not eliminated by using diets low in choline or supplemented with methionine, the first limiting amino acid in soybean protein.

Addition of fats to casein diet

In agreement with results obtained in other laboratories, addition of corn oil to the casein diet largely prevented the rise in plasma cholesterol (Fig. 3). Slightly lower values were also obtained when corn oil was added to commercial diet or to the diet containing soy protein isolate. On the other hand, butter seemed to have no consistent effect on plasma cholesterol levels when added to these diets.

Influence of carbohydrates

The influence of different carbohydrates was investigated by feeding them in a basic low fat diet with casein as source of protein and the results are shown in Figure 4. Wheat starch gave the highest average plasma cholesterol level and potato starch the lowest, with intermediate values for corn starch, rice starch and the three sugars tested. In another set of experiments, dextrose, wheat starch and potato starch were again fed in a casein diet and also in a similar diet with soy protein isolate replacing the casein (Fig. 5). As before, potato starch prevented the hypercholesterolemia normally obtained with the *casein diet*, but the values obtained with the soy protein diet were all low, regardless of the carbohydrate used.

DISCUSSION

FROM THESE RESULTS it appears that cholesterol-free semisynthetic diets only produce a hypercholesterolemic response in rabbits when they contain protein derived from an animal source. Even when this requirement is fulfilled, the response can be prevented by unsaturated fat such as corn oil or by carbohydrate such as potato starch. It is possible that the response may also be influenced by other components of the diets, but in our experience, the hypercholesterolemia was not prevented by replacing the salt mixture with one specially recommended for rabbits (Gaman et al., 1970) or by adding various sources of roughage such as wood fibre, wheat straw or oat hulls.

The fact that animal protein preparations appear to be primarily responsible for the hypercholesterolemia does not necessarily mean that the effect is due to protein per se, since even the best preparations contain materials other than protein. In order to explore this further, feeding trials were carried out with enzyme hydrolysates of casein and soybean protein and with mixtures of amino acids made up to simulate the compositions of these two types of protein. The enzyme hydrolysates gave results very similar to those obtained with the intact proteins. Results with the amino acid mixtures were less clear-cut, but the average plasma cholesterol level obtained with the mixture corresponding to casein was higher than that obtained with the mixture corresponding to soybean protein. It is obviously desirable to carry out more experiments along these lines in order to obtain further clarification of the problem.

Although these semisynthetic diets were generally well accepted by the rabbits, the growth rates were usually lower than those obtained with commercial laboratory chow diets. There was no apparent relationship, however, between growth rate and degree of hypercholesterolemia produced by the diets. Some hypercholesterolemic diets gave relatively good growth and others gave poor growth and the same could be said for semisynthetic diets which failed to produce a hypercholesterolemic response.

There also appeared to be no clear relationship between plasma cholesterol levels and rates of biosynthesis and degradation of cholesterol. Acetate incorporation into cholesterol by liver slices was generally lower on semisynthetic diets than on commercial diets and addition of casein to commercial diet appeared to inhibit incorporation, but the results were rather variable and did not show a close correspondence to observed plasma cholesterol values (Carroll, 1971). It was also found that rabbits on semisynthetic diets oxidized 26-^{14}C-cholesterol to $^{14}CO_2$

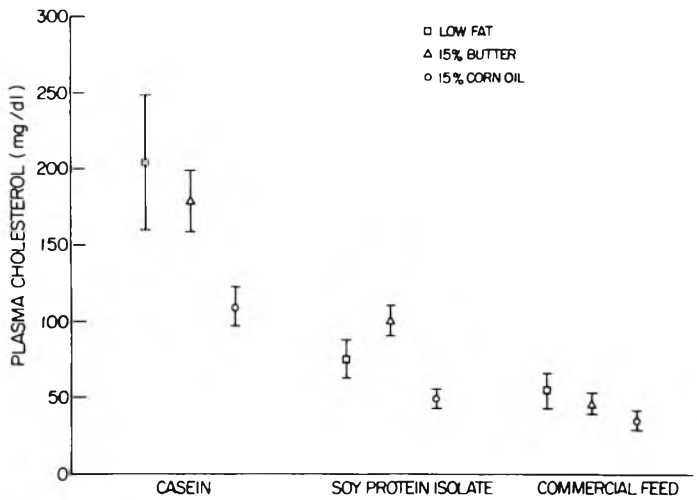

Fig. 3—Effect on total plasma cholesterol in rabbits of adding 15% by weight of butter or corn oil to semisynthetic or commercial diets. Values shown are Mean ± S.E.M. for groups of five to seven rabbits fed for 28 days. (See Table 1 for composition of diets.)

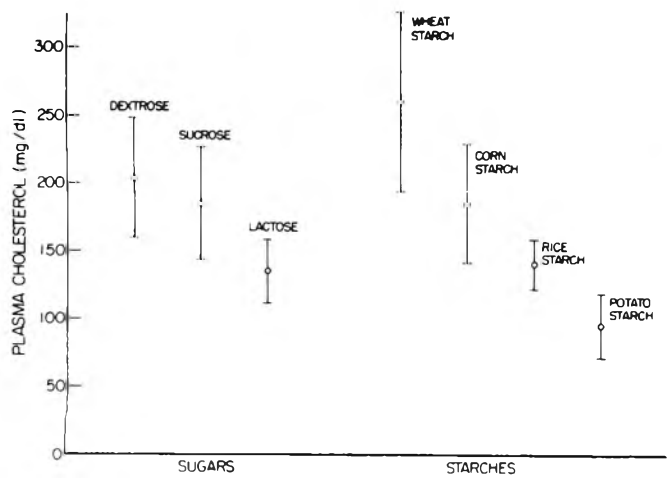

Fig. 4—Plasma total cholesterol levels in rabbits fed low fat, cholesterol-free semisynthetic diets containing different carbohydrates. Values shown are Mean ± S.E.M. for groups of five to six rabbits fed for 28 days.

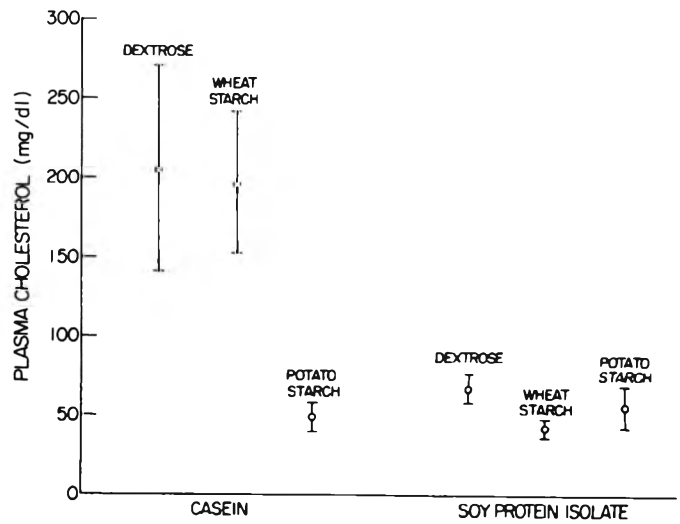

Fig. 5—Effect on plasma total cholesterol in rabbits of feeding different combinations of protein and carbohydrate in low fat, cholesterol-free semisynthetic diets. Values shown are Mean ± S.E.M. for groups of five rabbits fed for 28 days.

more slowly than rabbits on commercial diets (Suria, 1970), but rates of oxidation were similar on semisynthetic diets containing either casein or soybean protein, which gave quite different levels of plasma cholesterol. The biochemical basis of this difference may be related to the metabolism of components of plasma lipoproteins other than cholesterol.

Effects of low cholesterol semisynthetic diets in other animal species

Studies on factors affecting plasma cholesterol levels in experimental animals have as their ultimate goal the control of hypercholesterolemia and prevention of atherosclerosis in humans. It is therefore of interest to know whether results obtained by feeding cholesterol-free diets to rabbits are also applicable to other animal species and to humans.

Malmros and Sternby (1968) found that dogs developed a marked hypercholesterolemia on a semisynthetic diet containing hydrogenated coconut oil even when no cholesterol was added, and microscopic examination of the coronary arteries after a year showed some atherosclerotic changes. Robertson et al. (1972) confirmed the hypercholesterolemia in dogs on this diet, but observed no intimal changes in the cardiovascular system after 4 months feeding. They also cited studies by Malmros in which dogs on the diet for as long as 30 months failed to show arterial lesions.

Pigs maintained on cholesterol-free semisynthetic diets containing no fat, 10% beef tallow or 10% maize oil from weaning to 200 lb live weight failed to develop a significant hypercholesterolemia (Howard et al., 1965) and the diet did not appear to be atherogenic (Gresham et al., 1964).

Squirrel monkeys (Clarkson et al., 1969) and baboons (Howard et al., 1967) tend to become hypercholesterolemic in captivity and have more atheromata than their counterparts living in the wild, but it is not certain whether this difference is due to diet or to other environmental factors. Malmros et al. (1965) investigated the effect of low cholesterol semisynthetic diets on cynomolgus monkeys, using diets containing 17% by weight of hydrogenated coconut oil or corn oil respectively. Over a period of 137 wk the mean serum cholesterol rose from 125 to 200 mg/100 ml on the coconut oil diet and from 100 to 157 mg/100 ml on the corn oil diet.

Corey et al. (1974) have recently reported the results of experiments in which four different species of monkeys (squirrel, cynomolgus, cebus and spider) were fed various semisynthetic diets containing no cholesterol or levels of 0.1–0.2% cholesterol. Most of the dietary changes related to the fat and cholesterol content of the diets and hypercholesterolemic responses were obtained with both coconut oil and cholesterol, although there was considerable variation in the response of different species. In one experiment with spider monkeys, the serum cholesterol tended to rise as the carbohydrate was changed from dextrose to dextrin and then to sucrose in diets containing 0.1% cholesterol.

Howard et al. (1967) maintained seven baboons on a semisynthetic diet containing 20% beef tallow for 9 months and found that two of the animals had plasma cholesterols above the normal range, but at autopsy after 18 months only a small amount of arterial disease was seen. Strong and McGill (1967) fed baboons for 2 yr on semisynthetic diets containing different levels of cholesterol (0.01 and 0.5% by weight) and casein (10 and 25% by weight) with either saturated or unsaturated fat (22% by weight). Although the dietary cholesterol seemed to have the greatest effect on serum cholesterol and degree of atherosclerosis, animals on the low cholesterol, high protein, saturated fat diet all showed an increase in serum cholesterol (+ 20.8 mg/100 ml compared to + 29.6 mg/100 ml on the corresponding high cholesterol diet). Animals on the low cholesterol, high protein diets also tended to have more of the aortic intimal surface involved with sudanophilic lesions than animals on the corresponding low protein diets.

Kritchevsky et al. (1974) have recently reported on studies in which baboons were maintained for a year on cholesterol-free semisynthetic diets containing either glucose, fructose, sucrose or starch as source of carbohydrate. Serum cholesterol levels rose approximately 35% in all groups and at autopsy fatty streaks were observed in the aortas of animals on each of the four diets.

Role of diet in hypercholesterolemia and atherosclerosis in humans

Discussions of the role of diet in hypercholesterolemia and atherosclerosis in humans have concentrated mainly on dietary fat and sterols (Ahrens, 1957; Kinsell, 1963; Connor, 1968; Connor and Connor, 1972; Mattson et al., 1972) and recommendations aimed at reducing the incidence of cardiovascular disease typically include reduction of the intake of saturated fats and cholesterol, with substitution of polyunsaturated fats (see AMA, NAS-NSC Joint Statement of Diet and Coronary Disease, 1972; American Health Foundation Position Statement on Diet and Coronary Heart Disease, 1972). Nevertheless, while it is generally agreed that polyunsaturated fats depress the level of serum cholesterol to some extent, the role of saturated fats and dietary cholesterol in hypercholesterolemia and atherosclerosis remains a very controversial subject (Reiser, 1973; Keys et al., 1974).

Dietary carbohydrate can alter the level of serum triglycerides (Ahrens et al., 1961; Anderson, 1967; DenBesten et al., 1973) but does not appear to have much influence on serum cholesterol in humans (Grande, 1967). It has been suggested that sucrose consumption is a factor in the etiology of ischaemic heart disease (Yudkin, 1964; 1967; 1972) because of the positive correlation between sugar intake and mortality from atherosclerotic heart disease in different countries, but this hypothesis has not been widely accepted (McGandy et al., 1967; Paul et al., 1968; Walker, 1971; Keys, 1971; Connor and Connor, 1972).

Animal protein intake also shows a strong positive correlation with mortality from cardiovascular disease (Yudkin, 1957; Connor and Connor, 1972) and there is evidence that reducing the quantity and/or quality of dietary protein has a hypocholesterolemic effect in humans. The cholesterol-lowering effect of the low-protein, low-fat diet introduced by Kempner (1948) for treatment of hypertension is well known, and Olson et al. (1958) consider that the observed decrease in serum cholesterol cannot be entirely accounted for by the low-fat content of the diet. It is also known that serum lipids are low in protein deficiency states such as kwashiorkor and marasmus, perhaps because of decreased hepatic synthesis of the protein moiety of plasma lipoproteins (Truswell and Hansen, 1969). The serum cholesterol in children with kwashiorkor can be restored to normal levels by treatment with skim milk or various fat-free amino acid or protein formulas, provided they show a good clinical response to treatment (Schendel and Hansen, 1958). Malnourished adults also respond to dietary protein with a similar increase in serum cholesterol to normal levels (Tripathy et al., 1970).

Olson et al. (1958) observed a marked decrease in serum cholesterol of subjects transferred from a diet containing 100g of animal protein to an isocaloric, isofatty (50g of butterfat) diet containing 25g of vegetable protein derived from cereals, rice and legumes. Walker et al. (1960) reported that a group of young women consuming a diet containing 50g of protein derived mainly from vegetable sources had lower serum cholesterol levels than another group on a similar diet containing 50g of animal protein.

Hodges et al. (1967) observed an average drop of about 100 mg/dl in the serum cholesterol of six male subjects fed a diet based on soybean protein and containing virtually no animal fat and no cholesterol. Although part of this fall in serum cholesterol can be attributed to changes in dietary fat and cholesterol, there appeared to be a difference between the predicted and observed decrease, which may be due

to dietary protein (Keys and Hodges, 1967). Recent studies by Rickman et al. (1974) have shown that a diet recommended for weight reduction, consisting almost entirely of protein and animal fat, with carbohydrate contributing only about 2% of total calories, raised the level of serum cholesterol in all subjects studied. It seems possible that part of this response may be due to the protein component of the diet.

In contrast to the above studies, other workers have failed to observe any effect on serum cholesterol levels as a result of changing the amount or type of protein in the diet (Keys and Anderson, 1957; Anderson et al., 1971). Thus, as in the case of other dietary components, agreement is lacking on the role of dietary protein in the control of serum cholesterol levels.

Olson and his associates have attempted to throw more light on this problem by using amino acid formula diets (Olson et al., 1970a, b; Garlich et al., 1970). The amino acid mixtures contained the eight essential amino acids at levels of three times the tentative daily requirement, plus a source of nonessential nitrogen to keep the formula diets isonitrogenous with the control diet. In one diet, nonessential nitrogen was provided by glycine plus ammonium acetate and in another diet by glutamic acid. Some experiments were also carried out with a mixture containing nonessential amino acids in the proportions present in the control diet. A marked decrease in serum cholesterol was obtained with the diet containing glutamic acid but not with the other formula diets. This was primarily due to a fall in S_f 0–12 β-lipoproteins and was associated with decreased rate of entry of cholesterol into the plasma pool (Olson et al., 1970a). The effect may be related to an imbalance of amino acids but addition of glutamic acid to a normal diet had a negligible effect on serum lipids. Investigation of plasma free amino acid concentrations in subjects on the different diets showed some differences, but it did not appear that the cholesterol lowering effect was due to altered availability of amino acids to the liver for apoprotein biosynthesis (Garlich et al., 1970).

CONCLUSIONS

FROM THE FOREGOING discussion, it is evident that dietary protein and carbohydrate can affect serum lipid levels in humans as well as in other animal species and may also influence the development of atherosclerotic lesions. It is difficult to say whether these effects play a significant role in the etiology of cardiovascular disease in humans, but the experimental and epidemiological data now available indicate the desirability of further studies on nonlipid components of the diet in relation to atherosclerosis.

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JFS BASIC SCIENCE

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THERMAL EFFECTS ON THE LENGTH OF SARCOMERES IN MUSCLES HELD AT DIFFERENT TENSIONS

INTRODUCTION

THE RELATIONSHIP between the length of sarcomeres and the tenderness of meat has been well established since the initial observations of Locker (1960). However, the accumulation of data to establish this relationship has usually been from isolated unheated myofibrils, whereas tenderness values were obtained from heated muscles. Bendall and Voyle (1967) and Goll (1970) have cautioned on the advisability of such comparisons between unheated and heated muscles. Sarcomere length decreases when muscle is heated (Aronson, 1966; Dubé et al., 1972). The extent of the decrease may be dependent on the initial unheated sarcomere length as shown by Hegarty and Allen (1972) in turkey muscles.

The use of myofibrils for sarcomere length measurements may be an additional variable to avoid for the following reasons. Goldspink (1970) reports that a large fiber in mouse skeletal muscle can contain about 1250 myofibrils. Since 20–50 myofibrils from a sample are usually used for sarcomere length determinations in muscle tenderness studies, it is conceivable that a number of myofibrils from the same fiber may be measured in a sample. Distribution curves for sarcomere length values within muscles from farm (Hegarty and Allen, 1972) and laboratory animals (Hegarty and Hooper, 1971) illustrate the wide variation in sarcomere lengths obtained from isolated, unfixed and unheated fibers. Furthermore, variation in sarcomere length may occur along a single fiber due to postmortem handling practices. This effect can be more easily detected in the isolated whole fiber (Hegarty et al., 1973).

A further unstudied variable in the effect of heat on sarcomere length is the degree of restraint on the sarcomere to shorten when heated. Hostetler and Landmann (1968) concluded that free isolated fibers shorten when heated due to changes in the muscle proteins. However, they state that the heat effect on free floating fibers may bear little relation to the effect produced when an infinite number of fibers are heated, as in the case of a piece of meat.

Heat effects on collagen may be a factor in determining the final heated sarcomere length. High temperatures cause a shortening and disintegration of collagen molecules (Idson and Braswell, 1957). This presumably can have an effect on sarcomere length depending on whether the muscle is still attached by the tendons to the bones or whether the muscle is dissected free. Since meat is heated both with the muscles attached to the bones and also dissected free, it was considered important to study the effect of heat on sarcomere length for both these states. The effect of heat on isolated individual fibers was also investigated.

The muscles studied were the bovine psoas major, the semitendinosus and pectoralis major (breast) muscle in the turkey and the biceps brachii muscle in mice. The mouse was chosen as a type of model system because the fiber morphology has been well established (Rowe, 1967), and because of the ease in obtaining representative samples of the whole muscle. Furthermore, the effect of heat on rigor-stretched avian muscle (Hegarty and Allen, 1972) could be repeated on the more easily studied mouse muscle. Only the bovine psoas major muscle was studied because Hooper and Hegarty (1973) and further observations in this laboratory indicated a high content of passively contracted fibers in other bovine muscles. It is difficult to obtain accurate sarcomere length values on fibers that have undergone passive contraction.

MATERIALS & METHODS

SIX ADULT STEERS were slaughtered and suspended by conventional methods. The psoas major muscle was dissected after aging for 7 days at 2°C. Samples approximately 6 cm × 4 cm were cut from each muscle and used immediately in the studies described below.

Six adult turkeys were killed by placing the birds in an inverted metal cone to prevent excessive movement of the breast and limb muscles. The birds were exsanguinated by cutting the carotid artery and studied without scalding or plucking. The pectoralis major and semitendinosus muscles were dissected immediately from one side of the bird and allowed to go into rigor mortis at 20°C. The contralateral muscles entered rigor mortis at 20°C while attached to the bones. Previous studies showed that rigor mortis occurs by 6–8 hr postmortem (Hegarty and Allen, 1972). Ma et al. (1971) reported a wide variation (87–461 min) in rigor completion times for the pectoralis major muscle in turkeys restrained during slaughter. Muscles were then stored for a further 16–18 hr at 2°C. All of the beef and turkey muscle samples described above were placed in polyethylene bags, heated in a water bath and individual samples studied at 10°C intervals from 40–90°C. Internal temperature of these samples was measured by a thermocouple temperature recorder.

In a second group of six turkeys the pectoralis major muscle was dissected pre-rigor and the contralateral muscle dissected in rigor mortis. The muscle was cut into strips (approx 8 cm thick), placed in polyethylene bags and allowed to enter rigor mortis at 20°C, 2°C and −20°C. The frozen muscle was thawed at 20°C. All of these muscle strips were then heated in the manner described above to an internal temperature of 82°C. The semitendinosus muscle from these turkeys was sampled pre- and post-rigor to study the effect of heat on sarcomere length in samples of different thickness. After rigor onset, strips approx 6 cm and 0.5 cm in thickness were heated to 82°C.

Adult mice were killed by ether anaesthesia. One fore-limb of each animal was stretched forward maximally immediately postmortem and pinned at 180° from the trunk of the animal. The contralateral limb was pinned in a

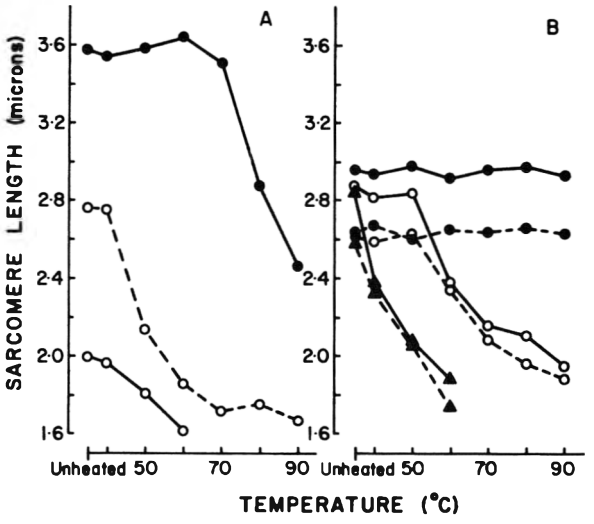

Fig. 1—Temperature effect on the length of sarcomeres in rigor muscles: (A) bovine psoas major ●——●; turkey semitendinosus, isolated fibers, dissected in rigor mortis ○– – –○; dissected pre-rigor ○——○; (B) mouse biceps brachii muscle, stretched pre-rigor ——— and in rigor mortis – – –, and then heated in situ (●), dissected from the bones (○) and isolated fibers (▲).

folded position. After rigor mortis (4 hr postmortem), the folded limbs were moved into a stretched position. Six animals were studied at each temperature within each treatment. The biceps brachii muscle was heated either with the limb intact, with the muscle attached to the bone but all surrounding muscles removed, with the muscles dissected off the bone and free of tendons, or with isolated individual fibers. These mouse muscle samples were heated in a water bath for 10 min at temperatures of 10°C intervals from 40–90°C.

Isolated muscle fibers were heated in flat-bottom vials containing Ringer Locke solution. The temperature of the solutions was maintained at 10°C intervals from 40–90°C.

Individual fibers from all the muscles used in this study were separated and the length of sarcomeres measured as outlined by Hegarty and Allen (1972). Sarcomere length was determined by counting the number of A-bands along 100μ in each of 25 fibers from each muscle. Electron micrographs were obtained by the procedures outlined by Hegarty et al. (1973).

Statistical differences among treatments were tested by the t-test as outlined by Steel and Torrie (1960).

RESULTS & DISCUSSION

NO SIGNIFICANT change in sarcomere length was observed in a segment of bovine psoas major muscle until the muscle was heated to 70–80°C (Fig. 1A). Dubé et al. (1972) observed the decrease to occur between 60–70°C in this muscle but the subsequent pattern of sarcomere shortening was similar to that observed in the present study.

Sarcomere length in isolated fibers from pre-rigor and rigor-dissected turkey semitendinosus muscle decreased significantly when heated above 40°C. Ultimate sarcomere length values between 1.6–1.7μ were reached at 60°C in the rigor-dissected muscles. Termination of the graphs below 90°C in Figure 1 (A, B) indicate that no sarcomeres were visible. It must be stressed that sarcomere length values less than 2.0μ in the present investigation may be biased on the large side because sarcomeres were not observed in the more highly contracted fibers. Values less than 1.5μ were not observed and it was assumed that fibers devoid of cross-striations had shortened to values of less than 1.5μ. This assumption is based on the fact that the myosin (A-band) has a thickness of 1.35μ in the ox (Locker, 1959) and 1.4μ in the chicken pectoralis major muscle (Hagopian, 1970). Maximum overlap of the actin (I-band) and myosin would therefore give a sarcomere length of 1.35–1.6μ. In this situation there would be no contrast between the light I-band and the dark A-band, thereby making it impossible to count the A-bands under the light microscope. An example of a highly contracted myofibril from unheated turkey semitendinosus muscle with minimum contrast between the A- and I-band is shown in Figure 2A. Locker (1959) reported sarcomere lengths of 0.7μ in unheated ox muscle. This, however, was exceptional since most of the sarcomeres were 1.1μ or longer.

Aronson (1966) reported that isolated glycerinated rabbit fibers shortened to 40% of their initial length when heated to 70°C. However, the results of the present study show that both the magnitude and percentage decrease was dependent on the length of the unheated sarcomeres. In Figure 1A the sarcomere length in the isolated fibers of the turkey semitendinosus muscle, which was dissected after rigor mortis occurred, decreased to 60% of the initial length (2.77 to 1.72μ) when heated to 70°C. The isolated fibers from the contralateral muscle, which was dissected immediately postmortem, decreased to 80% of the initial sarcomere length (2.00 to 1.61μ) under the same conditions. Aronson (1966) showed that heat shortened both the A-band and I-band. Unpublished observations in our laboratory confirm this observation. Heating both the turkey semitendinosus and pectoralis major muscles to 82°C caused a decrease in A-band width from 1.5 to 1.0μ (unpublished observations). It is of interest to note that when the initial decrease in sarcomere length occurred in the bovine psoas major and isolated fibers from the rigor-dissected turkey semitendinosus muscles (Fig. 1A), dissected mouse biceps brachii muscle and the isolated fibers of the same muscle (Fig. 1B), the decrease in sarcomere length was about 0.5μ in all cases. Aronson (1966) does not refer to this observation but the same order of magnitude of sarcomere length decrease due to heat can be deduced from his data. It may be tentatively postulated that the initial decrease in the length of sarcomeres when heated may be due to a shortening of the A-band. Jacobson and Henderson (1973) report that the temperature for maximum change in the overall conformation (the melting temperature, T_m) for both myosin and actomyosin was 43 ± 2°C. Overall conformational changes were reversible below the T_m. This is interesting because in the present study sarcomere shortening occurred in the isolated fibers when heated between 40–50°C (Fig. 1A, B).

Ultrastructural differences observed in different heated muscles may be a variable in sarcomere length measurements on heated muscle. Heating to 82°C completely removed the thin filaments in the pectoralis major muscle of the turkey (Fig. 2B). The precipitated thin filaments are still visible in the semitendinosus muscle (Fig. 2C). This visual loss of thin filaments due to heat from its normal structural location in the sarcomere may facilitate the apparent decrease in sarcomere length as observed under the light microscope. The effect of heat on actin from different muscles is being investigated in this laboratory. The effect of heat on the actin filaments results in a greater contrast between light and dark bands. It is therefore easier to measure sarcomere length in heated muscle compared to unheated muscle under the light microscope, especially when the length of sarcomeres is between 1.6–2.0μ.

Because of the ease of sampling, mouse muscle was used to investigate the effect of heat on the length of sarcomeres in isolated fibers, dissected muscle and muscle attached to the bone. Shortening of sarcomeres in isolated mouse muscle fibers (Fig. 1B) occurred in a similar manner to that observed for the isolated turkey semitendinosus fibers (Fig. 1A). No sarcomeres were observed when the isolated mouse muscle fibers were heated above 60°C. Sarcomere length commenced to decrease between 50–60°C when the dissected muscle was heated. This is in contrast to the bovine psoas major where the shortening in sarcomere length due to heat was observed at 70–80°C (Fig. 1A). No effect of heat on sarcomere length was observed when the mouse muscle was heated while attached to the bone under the following conditions: (a) surrounding musculature intact; (b) surrounding musculature removed; (c) limb pinned in a stretched position; (d) limb in a relaxed position. This observation was contrary to the observations on rigor-stretched turkey semitendinosus muscle (Hegarty and Allen, 1972). An explanation for the difference is that all the fibers in the mouse biceps brachii muscle extend from tendon to tendon (Rowe, 1967) whereas the fibers interdigitate in the adult turkey semitendinosus muscle. Despite the fact that the dissected turkey semitendinosus muscle was held between clamps while heating (Hegarty and Allen, 1972), the sarcomeres, nevertheless, has a greater possibility to shorten due to heterogeneous distribution of tension in the fibers which is caused by some fibers not being secured at the ends of the sample. Weidemann et al. (1967) reported the same difficulty in heating strips of ox muscle. The mouse muscle experiments provide a good example of the inadvisability of using sarcomere length measurements from unheated muscle as predictors of heated sarcomere length values unless it is stated whether the muscle is heated while attached to the bone or dissected free.

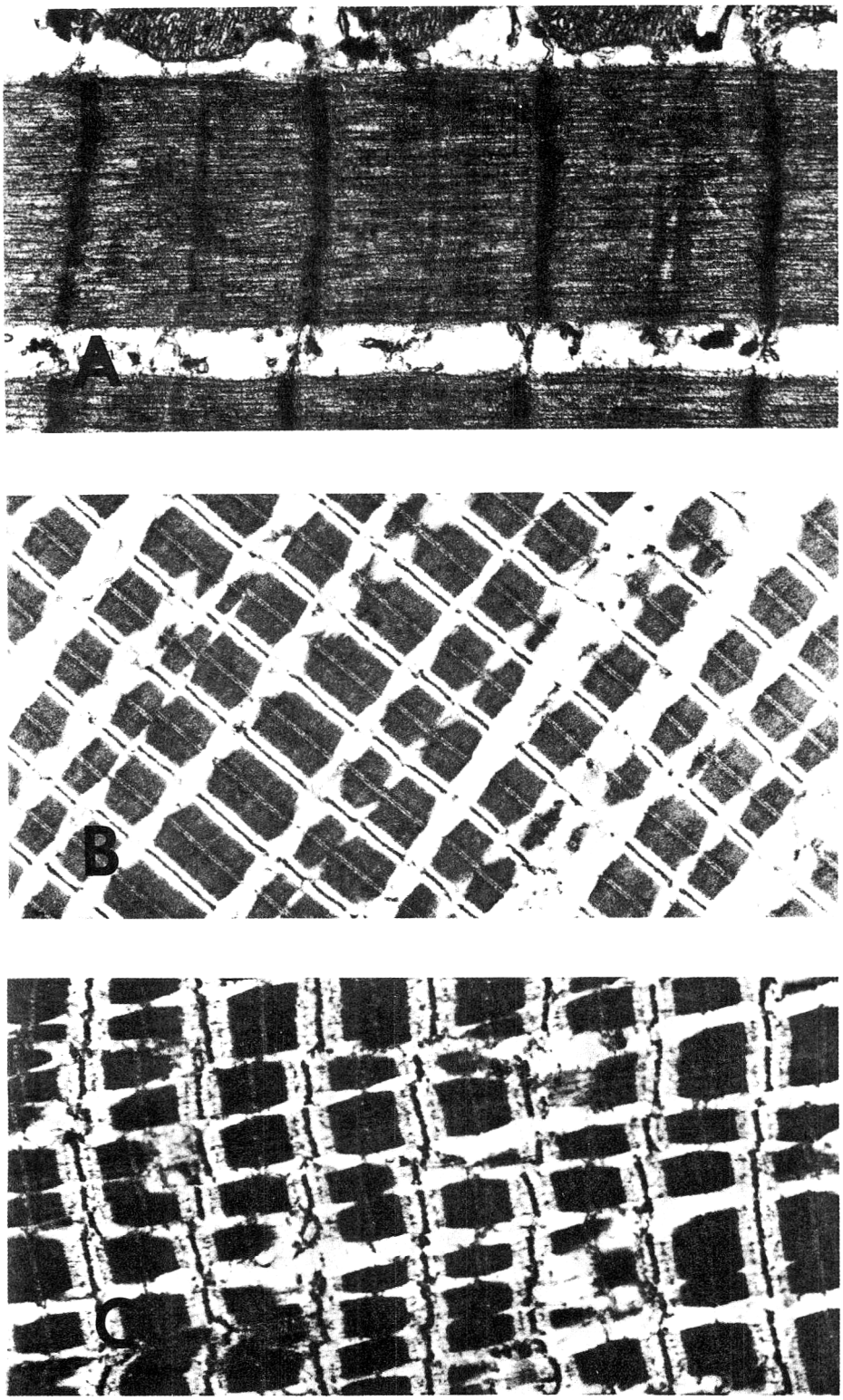

Fig. 2–Ultrastructure of turkey muscles: (A) unheated semitendinosus muscle in a highly contracted state, ≈21,000×; (B) pectoralis major heated to 82°C, ≈5,575×; and (C) semitendinosus muscle heated to 82°C, ≈6,900×. Note the differential heat effect on thin filaments in both muscles.

The above observations were applied to an investigation on the effect of heat on the length of sarcomeres in some practical situations (Table 1). The mean sarcomere lengths in turkey pectoralis major muscle, which was dissected immediately postmortem and allowed to enter rigor at either 20°C or 2°C, were not significantly different from the contralateral muscle which entered rigor under the same conditions while attached to the bone. This observation was made on both the unheated and heated muscles. These results are contrary to the observations of Welbourn et al. (1968) and Smith et al. (1969). Pre-rigor dissected muscle permitted to undergo thaw-rigor had significantly shorter sarcomeres in both the unheated ($P < 0.05$) and heated ($P < 0.01$) state.

Since the effect of heat caused a greater decrease in sarcomere length in individual isolated fibers than in the entire muscle (Fig. 1B), it was decided to examine the heat effect on thin strips of

Table 1–Effect of postmortem muscle treatment on the length of sarcomere in unheated and heated (82°C) turkey muscles (n=6)

Muscle	Treatment		Sarcomere length (μ)		
			Pre-rigor dissected		Rigor-dissected
Pectoralis major	Rigor, at 20°C	Unheated	1.98 ± 0.04	N.S.	2.09 ± 0.04
		Heated	1.75 ± 0.05	N.S.	1.81 ± 0.02
	Rigor, at 2°C	Unheated	1.97 ± .04	N.S.	2.08 ± 0.04
		Heated	1.80 ± 0.06	N.S.	1.81 ± 0.03
	Rigor, frozen/thawed	Unheated	1.90 ± 0.02	*	2.10 ± 0.08
		Heated	1.67 ± 0.03	**	1.83 ± 0.03
Semitendinosus[a]	Entire muscle minus	Unheated	2.00 ± 0.06	**	2.77 ± 0.22
	thin strip	Heated	1.72 ± 0.01	**	2.29 ± 0.09
	Thin strip of	Unheated	2.00 ± 0.06	**	2.77 ± 0.22
	above muscle	Heated	1.73 ± 0.01	**	2.16 ± 0.08

[a] The muscle entered rigor mortis at 20°C
* $P < 0.05$
** $P < 0.01$

muscle and to compare the results with values for the remainder of the muscle. There was no significant difference (Table 1), indicating that size of sample larger than approx. 0.5 cm thick is not a variable in determining sarcomere length in heated muscles. The effect of pre-rigor dissection of the semitendinosus muscle had a significant effect ($P < 0.01$) on the length of sarcomeres when compared to the rigor-dissected muscle in the unheated state. Though the length of the sarcomeres decreased when the samples were heated, the significance of the difference between the pre-rigor and rigor-dissected muscles was still maintained ($P < 0.01$).

Paul (1965) reported cracks in fibers after heating, and Voyle (1969) observed irregular sarcomere patterns when bovine muscle entered rigor mortis at 2°C. Neither of these phenomena was observed in the muscles used in this study. However the fibers from unheated and heated bovine longissimus dorsi, semitendinosus, biceps femoris and semimembranosus muscles showed a high degree of passive contraction ("kinking") with few observable sarcomeres. These observations were made on these muscles from the same animals that provided the psoas major samples for studies reported in this paper. Fibers in the psoas major did not exhibit any passive contraction.

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RELATIONSHIPS OF ELECTROPHORETIC PATTERNS AND SELECTED CHARACTERISTICS OF BOVINE SKELETAL MUSCLE AND INTERNAL TEMPERATURE

INTRODUCTION

INTERNAL end-point temperature is associated with palatability and acceptability of meat. Several authors have cited the importance and difficulties of relating changes in the sarcoplasmic protein fraction of skeletal muscle to properties of muscle as food (Goll et al., 1970; Hamm, 1966; Scopes, 1970).

Paul et al. (1966) heated samples of rabbit longissimus dorsi muscle 2–10 hr at 40–80°C. They found decreases in solubility of sarcoplasmic and myofibrillar proteins after 2 hr at 40°C, after 30 min at 45°C and by the time the tissue had reached 50°C. Higher temperatures and longer heating periods resulted in increases in protein soluble in 0.1N NaOH (defined as denatured protein). Laakkonen et al. (1970) investigated effects of low-temperature, long-time heating of three bovine muscles on changes in polyacrylamide gel electrophoretic patterns of the water-soluble proteins and juices from the muscles. Changes in electrophoretic patterns, i.e., the number and estimated intensity of stained protein bands, were evident with increased end-point temperature and time of heating. Slow-moving anodic proteins changed most rapidly when heated for various times at 37°, 45° and 60°C, resulting in a decrease in number and in estimated intensity of associated electrophoretic bands. Myoglobins and myoalbumins were altered significantly only by holding meat at 60°C. Perhaps those changes were caused by protein denaturation.

From data reported by Lawrie (1966), it is estimated that about 2/3 of the water-soluble materials in bovine muscle are sarcoplasmic proteins. No data were found in the literature relating internal temperature to specific alterations in sarcoplasmic proteins in skeletal bovine muscle. This study investigated: (1) effects of two internal temperatures (25° and 45°C) in semitendinosus muscle (ST) on electrophoretic patterns of heme proteins soluble in low ionic strength buffer; and (2) relationships between the color of the interior of raw and heated (25° or 45°C) ST muscle and spectrophotometric scans of electrophoretic gels containing sarcoplasmic proteins from the raw and heated muscle.

MATERIALS & METHODS

U.S. CHOICE grade beef ST muscles were obtained from a local wholesale meat purveyor as needed. Excess fat and connective tissue were trimmed from exteriors of the muscles. Two roasts similar in size and shape were cut from the center of each muscle. Roasts were assigned at random to heat treatments. Proximal and distal ends of the muscles were used for baseline data on raw meat.

Roasts were heated at 149°C in a gas, rotary-hearth oven to an endpoint temperature of either 25° or 45°C, as determined by a glass, red-spirit thermometer inserted into the center of the roast. Heat penetration rate (min/5°C) and cooking losses (percentage total, volatile and drippings) were determined for each sample.

Slices of the heated roasts (approx 1.3 cm thick) directly adjacent to the proximal and distal sides of the thermometer pathway were used to measure Gardner color-difference values or for extraction of the sarcoplasmic fraction. Areas of roasts next to those slices were ground and mixed, and aliquots were used to measure pH, total moisture and ether extract.

Color of the raw and heated muscle was measured using a Gardner Color Difference Meter standardized with a satin finish ceramic tile similar in color to the usual conception of the color of rare meat (Rd = 9.2; a = +20.5; b = +14.8). The center of a slice of meat was cut with a round cookie cutter and allowed to bloom for 5 min in air, then inserted in the Gardner Color Difference sample cup. An initial set of readings, and a second set, obtained after rotating the sample cup 90°, were averaged to obtain Rd, a^+ and b^+ values for each sample.

Sarcoplasmic proteins were extracted from raw and heated muscle at approximately the same time color-difference values were obtained. The extraction procedure was modified from that of Awad et al. (1968).

All equipment and buffers used in the extraction procedure were chilled. Muscle was ground twice; 30g ground muscle were homogenized with 60 µl sodium phosphate buffer (pH = 7.2, μ = 0.05) 1 min at high and 4 min at medium speed (medium = 6, high = 9, with 1 being the lowest speed and 11, the highest attainable). Residue on the blade of the homogenizer was rinsed into the homogenizer flask with 5 ml buffer. The homogenate was stirred for 15 min on a magnetic stirring plate set at medium speed, and then centrifuged at 3,000 rpm for 20 min at 4°C. The supernate was filtered once through glass wool and once through Whatman No. 42 filter paper.

The residue remaining in the centrifuge tube was extracted by homogenizing with 60 ml of buffer for 2 min at high speed and 4 min at medium speed. Residue on the homogenizer blade was rinsed into the flask and centrifuging and filtering were repeated. Extracts obtained for each treatment were combined, concentrated and dialyzed using a Bio-Fiber 50 Beaker Dialyzer. Concentration was achieved by passing a 20% solution of Polyglycol E-20,000 through the fibers while the sample remained outside the fibers. All extraction, concentration and dialysis procedures were carried out in a crushed-ice bath. Preliminary investigation showed no differences between electrophoretic patterns of the extracts before and after concentration and dialysis.

Nitrogen content of 0.5 ml of each extract was determined by the Biuret procedure (Layne, 1957). For each nitrogen determination, a standard curve was formulated using bovine serum albumin as the protein standard. Extracts were stored overnight at 4°C before electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (SDS). Preliminary investigation showed no differences in electrophoretic patterns before and after overnight storage.

Total moisture was measured by drying duplicate 10-g samples of ground raw or heated muscle at 121°C for 2 hr 45 min and 1 hr 45 min, respectively, in a Brabender Semiautomatic Moisture Tester. Duplicate pH measurements were made on slurries formed by blending 100 ml distilled-deionized water with 10g ground raw or heated muscle cooled to 25°C (Rogers et al., 1967). Ether extract was measured on samples obtained from muscle dried for moisture analyses. Samples (approximately 2g) were extracted with petroleum ether (low boiling) 16 hr on a Goldfisch extraction apparatus.

Proteins were separated, on the basis of molecular weight, by electrophoresis in SDS (highly pure grade) using a method modified from that of Weber and Osborn (1969). SDS was recrystallized from 95% ethanol, filtered and placed in a vacuum oven (736 mm Hg) overnight at room temperature to remove any residual ethanol. An amount of protein extract corresponding to 2 mg N was incubated in 0.5 ml of 0.01M phosphate buffer (pH 7.0, μ = 0.05, 1% in SDS and 0.02M in iodoacetamide) at 37°C for 2 hr. The incubation solution was prepared immediately before use. Approximate-

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ly 1 min before the end of incubation, 6 mg ± 0.5 mg sucrose crystals were added to the incubated mixture, and samples containing 0.2 mg N were applied to 7.5% polyacrylamide gels containing 0.1% SDS. Each extract was applied to four gels, and a current of 8 ma per gel was applied for 4 hr. Bromophenol blue mixed with buffer in the upper chamber of the electrophoresis apparatus was used as a marker to indicate the distance the fastest moving components migrated into the gel.

After electrophoretic separation, two gels from each sample were scanned in a photometric gel scanner (model UA 4 absorbance monitor, type 5 optical unit, Instrumentation Specialties Co., Lincoln, Nebr.) at 405 nm, the region of the Soret peak of myoglobin. An electronic digital integrator was employed to quantitate areas under selected absorbance peaks.

The distance polypeptides migrate into SDS gels is related to polypeptide molecular weight (Shapiro et al., 1967; Weber and Osborn, 1969). In a mixture of proteins of varying molecular weights, those with largest molecular weights migrate the shortest distances into polyacrylamide-SDS gels. Thus, electrophoretograms were used in this study to measure migration distances of selected absorbance peaks. All migration measurements were made in mm from the point representing the origin of the gel on the scan to a line perpendicular to baseline and passing through the highest point of absorbance of selected peaks (Fig. 1).

A third gel from each sample was stained with Coomassie blue as described by Weber and Osborn (1969), except that gels were stained for 1 1/2 hr and destained for 3 days. The fourth gel from nine replications was stained with the heme-detecting stain described by Owen et al. (1958).

Statistical analyses

Data for all measurements were analyzed by analysis of variance. When F-values were significant, least significant differences (LSD) at the 5% level of probability were calculated. Least squares Analysis of Data with Unequal Subclass Numbers (Anonymous, 1960) was used to analyze parameters with missing observations. Correlation coefficients were calculated for selected parameters. Bartlett's test of homogeneity of variance (Snedecor, 1956) was employed to detect significant variances among observations for selected parameters.

RESULTS & DISCUSSION

Heat induced changes in muscle

Values obtained for analyses of raw samples from the two ends of the ST muscle did not differ significantly for any parameter used to evaluate each muscle and sarcoplasmic extracts from those muscles (Table 1). Because those samples were obtained from different muscles and from opposite ends of a muscle, apparently, for the parameters investigated, the proximal and distal ends of the ST muscle varied little.

Heating, irrespective of end-point temperature, resulted in an increase ($P < 0.05$) in muscle pH. As internal temperature of muscle increased, total moisture decreased ($P < 0.05$) and volatile and total cooking losses increased ($P < 0.05$). Percentage drip cooking losses did not differ significantly between heat treatments. Hamm (1966) attributed loss of moisture from muscle during heating to coagulation of myofibrillar proteins by heat denaturation, with resultant loss of water-holding capacity.

Percentage drip and total cooking losses from muscle heated to both end-point temperatures varied ($P < 0.01$) among observations at each temperature. Variance was greater ($P < 0.01$) at 45°C than at 25°C (Table 2).

Percentage ether extractable substances (dry-weight basis) increased ($P < 0.05$) with heating of muscle to each internal end-point temperature (Table 1). Because the raw samples did not differ significantly in lipid content, the effect of heat apparently was responsible for the increased percentage lipid in heated samples. Woolsey and Paul (1969) explained that heating ST muscles rendered lipid in the muscle more susceptible to extraction. They extracted a greater ($P < 0.01$) percentage crude fat from ground samples of roasts heated to 58°C than from samples of unheated roasts. Also, percentage ether extractable lipid was greater ($P < 0.01$) for muscles heated to an internal temperature of 58°C in a 163°C oven than for those heated in a 218°C oven.

Heating the ST muscle changed its Gardner color-difference values for reflectance (Rd), redness (a^+) and yellowness (b^+). Reflectance of muscle heated to 25°C did not differ significantly from that of raw muscle, but reflectance of muscle heated to 45°C was greater ($P < 0.05$) than that of raw muscle and muscle heated to 25°C. Values for redness and yellowness did not show clear cut differences between raw and heated samples; a^+/b^+ ratios did not vary significantly among treatments. However, for the 45°C treatment, the a^+/b^+ ratio was slightly lower than that for raw muscle or muscle heated to 25°C. According to Snyder (1964), a decrease in the a^+/b^+ ratio indicates formation of metmyoglobin.

The rate heat penetrated muscles did not vary significantly between treatments for the interval 20–25°C (Table 1). That temperature range was the only common temperature range for which rate of heat penetration was measured for both heat treatments.

Electrophoretic evaluation of sarcoplasmic extracts

Protein content of sarcoplasmic extracts (mg N/0.5 ml) from raw samples did not differ significantly between the proximal and distal ends of the muscle. Extractable protein decreased ($P < 0.05$) in muscle heated to 25°C and decreased ($P < 0.05$) further between 25° and 45°C (Table 1). During preliminary investigation of various internal end-point temperatures, yield of the sarcoplasmic fraction, based on extraction, was impaired greatly when the internal temperature of the muscle was above 45°C.

A general protein stain (Coomassie blue) was used to observe differences in electrophoretic patterns of sarcoplasmic extracts from raw and heated muscle. In this study, the staining system of Weber and Osborn (1969) yielded more clearly stained gels than did that recommended by Shapiro et al. (1967), who fixed gels in sulfosalicylic acid before staining with Coomassie blue. Williams and Gratzer (1971), who reported precipitation of Coomassie blue on SDS gels, suggested that the anionic nature of SDS was responsible for the adverse reaction. In this study, no such difficulties were encountered in using that stain.

Typical pairs of electrophoretograms that demonstrate effects of heating muscle on sarcoplasmic extracts are shown in

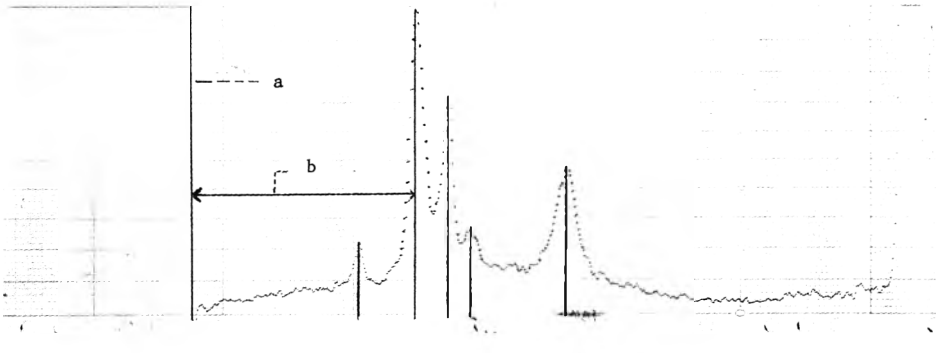

Fig. 1–Spectrophotometric scan (405 nm) of a polyacrylamide disc gel demonstrating measurement of distances of migration of selected absorbance peaks. The anode is at the right side of the scan and all succeeding electrophoretic scans. (a) Line representing origin of gel in a quartz electrophoresis tube and the point from which migration distances were measured. (b) One of five distances of migration measured for electrophoretic scans of gels.

Figures 2 and 3. A decrease in the number and intensity of mainly the slowest migrating (largest) protein components of sarcoplasma was associated with heating muscle to an internal temperature of 25°C (Fig. 2); the decrease in number and intensity of protein components in the sarcoplasmic extract from muscle heated to 45°C was more extensive, as evidenced by disappearance of relatively more components from the 45°C extract (Fig. 3).

To observe the number of heme pigment bands present in gels after electrophoresis, two staining systems were investigated. Hemoproteins catalyze oxidation of benzidine and o-dianisidine in the presence of hydrogen peroxide; thus, those dyes can be used to detect hemoproteins (Owen et al., 1958; Randall and MacRae, 1967; Smithies, 1959). Stransky and Srch (1967) used a benzidine stain and an o-dianisidine stain to detect hemoglobin and myoglobin. respectively, on thin-layer Sephadex plates. Setser (1971) used a benzidine stain to detect myoglobin in electrophoretic gels. In this study, the staining procedure of Setser (1971) resulted in a black precipitate that covered gels so that no bands were visible. However, the o-dianisidine staining system of Owen et al. (1958) was effective, and resulted in clearly stained bands of heme protein within gels, but those stained gels did not photograph well. Figure 4 represents the pattern obtained by using the o-dianisidine stain on a typical SDS gel containing sarcoplasmic extract.

The number of bands stained with o-dianisidine did not vary significantly among treatments (Table 1). However, usually more bands were visible in gels containing sarcoplasmic extracts from muscle heated to 25° or 45°C than in gels containing sarcoplasmic extracts from raw samples. Number of visible bands varied more ($P < 0.01$) for both heat treatments than for raw samples (Table 2). The appearance of several heme bands supports the findings of other researchers that bovine myoglobin (Mb) is heterogeneous (Quinn et al., 1964; Quinn and Pearson, 1964; Laakkonen et al., 1970; Randall and MacRae, 1967; Satterlee and Snyder, 1969). The number of components detected in those studies ranged from 3–9. Appearance of more heme-positive bands in gels containing sarcoplasmic extract from heated muscle than in gels containing extract from raw muscle is puzzling. Heat-induced alterations in molecular size through ligand exchange, or in conformation of pigment molecules, could result in varied resistance of molecular species to movement through gels and/or varied binding of SDS to proteins. Either occurrence could influence the number of visible heme bands.

During preliminary studies, use of 2-mercaptoethanol to disrupt proteins resulted in complete loss of Soret absorbance of the proteins under study. Moreover, Atassi (1970) reported absence of cysteine and cystine in bovine Mb, thus eliminating the possibility of disulfide

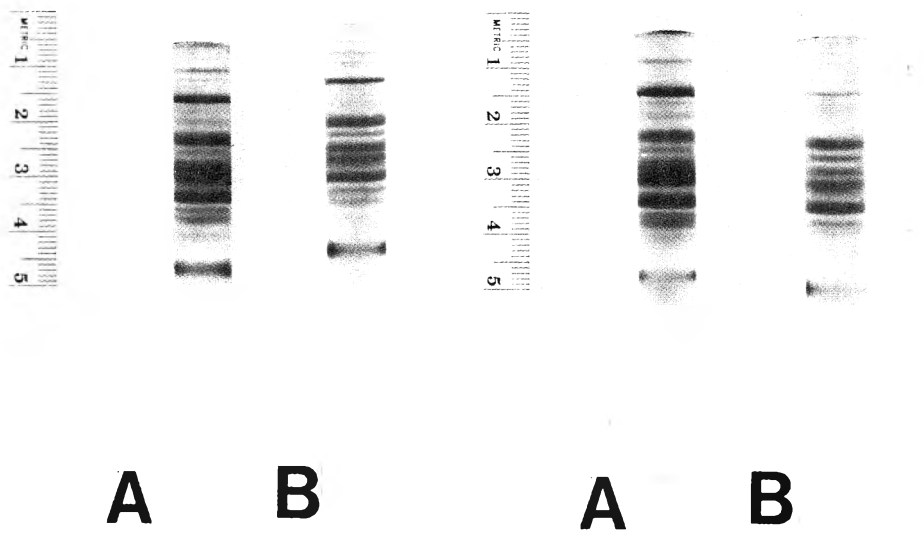

Fig. 2—Typical electrophoretograms of stained SDS-polyacrylamide gels containing sarcoplasmic extract from raw muscle (A) and muscle heated to 25°C (B). Gels were stained with Coomassie blue. The anode is at the bottom of the electrophoretic gel tube in this and all subsequent figures of electrophoretic gels. Bromophenol blue marker dye is not visible.

Fig. 3—Typical electrophoretograms of stained SDS-polyacrylamide gels containing sarcoplasmic extract from raw muscle (A) and muscle heated to 45°C (B). Gels were stained with Coomassie blue. Bromophenol blue marker is not visible.

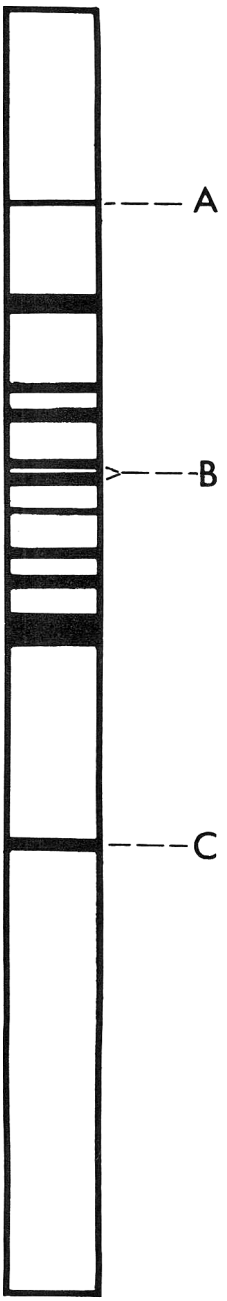

Fig. 4—Pattern visible on staining an SDS-polyacrylamide gel with o-dianisidine after electrophoresis (1.5 cm in figure = 1 cm of gel). (A) Origin of gel in quartz tube. (B) Heme bands representing peaks 2 and 3 on electrophoretic scans. (C) Bromophenol blue marker dye.

bonds. Preliminary work indicated no observable differences in electrophoretic patterns of reduced and non-reduced proteins in stained gels containing sarcoplasmic extract from raw or heated muscle. Dunker and Rueckert (1969) reported that proteins reduced with 2-mercaptoethanol and nonreduced proteins with greater than 10,000 molecular weight varied little in mobility in SDS-polyacrylamide gels. Therefore, 2-mercaptoethanol was not used in the final experiment.

Bernofsky et al. (1959) suggested that co-precipitation of Mb with other water-soluble proteins present in meat accounted for denaturation of that pigment at temperatures lower than those necessary to effect denaturation of Mb in pure solutions of the same pH. Ledward (1971) and Draudt (1969) supported the role of other water-soluble proteins in denaturation of Mb in model systems and muscle extracts heated under various time-temperature programs. Draudt (1969) reported that, depending on the nature of the ligand at the sixth coordinated position of the heme, Mb in meat precipitated at temperatures 15–30°C below the temperature required in a pure system of the same pH. In light of that information, combined with difficulties in extracting proteins from muscle heated to temperatures above 45°C, temperatures of 25° and 45°C were used, expecting to obtain information on heat-induced alterations in Mb before it precipitated appreciably.

Ligands at positions 5 and 6 of Mb, other water-soluble nonheme proteins present in meat and predenaturation conformational changes in the hematin environment have been implicated as contributors to the heterogeneity of cooked meat pigments. Ledward (1971) suggested that the heme complexes in cooked meat are denatured hemoproteins in which the protein can be any of several of the denatured proteins present.

Separation of proteins by polyacrylamide gel electrophoresis in the presence of the anionic detergent SDS should be governed by molecular weight differences among proteins. SDS, a powerful detergent, reportedly disrupts native secondary and tertiary structure (hydrogen and hydrophobic bonds) of proteins, and then, binds to proteins in such a manner that any native charge that the protein possesses generally affects protein migration distances minimally (Shapiro et al., 1967; Shapiro and Maizel, 1969). Weber and Osborn (1969) and Dunker and Rueckert (1969) reported data that indicated close agreement between molecular weight values for Mb (17,400) re-

Table 1—Treatment means,[a] F-values and LSD values for parameters used to evaluate raw muscle, muscle heated to two end-point temperatures and sarcoplasmic extracts from those muscles.

Parameter	Treatment				F-value	LSD[c]
	Raw 1[b]	25°C	45°C	Raw 2[b]		
Intact muscle						
pH	5.52d	5.57e	5.59e	5.52d	8.93***	0.036
Total moisture (%)	72.42d	69.84e	66.56f	72.57d	46.79***	1.19
Cooking losses (%)						
Volatile	–	4.35	8.57	–	241.32***	0.61
Drip	–	0.77	1.34	–	4.26[ns]	–
Total	–	5.46	10.15	–	84.99***	1.14
Ether extract (%)	12.17d	15.63e	19.43f	11.92d	18.75***	2.35
Color-difference, Gardner						
Reflectance (Rd)	9.6d	10.2d	12.8e	9.9d	9.44***	1.37
Redness (a+)	17.9d,e	15.8f	16.8e,f	19.2d	7.43***	1.57
Yellowness (b+)	9.4d	9.3d	10.6e	10.1d,e	3.52*	0.95
Ratio (a+/b+)	1.9	1.8	1.6	1.9	1.52[ns]	–
Rate of heat penetration (20° to 25°C)	–	3'57"	4'57"	–	2.20[ns]	–
Electrophoretic data (Sarcoplasmic extracts)						
Number of heme bands stained with o-dianisidine	6.0	7.0	7.0	6.0	1.94[ns]	–
Migration distances of selected absorbance peaks (cm from origin)						
Peak 1	2.72d	2.75d	2.87d,e	2.99e	2.97*	0.21
Peak 2	3.74	3.78	3.90	4.07	2.58[ns]	–
Peak 3	4.23	4.19	4.43	4.59	2.41[ns]	–
Peak 4	4.66	5.08	4.88	4.91	1.96[ns]	–
Peak 5	6.00	6.22	6.37	6.46	1.39[ns]	–
Integrals corresponding to selected absorbance peaks						
1	1,556.0d	2,024.0d	3,212.0e	1,325.0d	9.05**	806.0
2	24,278.0	20,757.0	27,470.0	20,853.0	1.03[ns]	–
3	9,419.0	9,530.0	18,816.0	6,418.0	6.70**	–
4	994.0	1,887.0	3,432.0	857.0	0.003[ns]	–
5	2,375.0	2,632.0	13,642.0	1,958.0	9.98***	–

[a] Differences between means followed by the same letter are not significantly different at the 5% level.
[b] Raw 1 and Raw 2 indicate treatments used as a reference point for muscle heated to 25° or 45°C, respectively.
[c] LSD, Least significant difference at the 5% level
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; [ns]not significant

ported in the literature and values obtained experimentally using polyacrylamide-SDS electrophoresis. However, Tung and Knight (1972) reported anomalous migratory behavior of Mb in 10% acrylamide-SDS gels. Mb migrated as though its molecular weight were 14,000. They suggested that the intrinsic charge of Mb, an unusual conformation of Mb in SDS or an interaction of those two factors was responsible for the electrophoretic behavior of Mb. In light of their findings, Tung and Knight (1972) stated that two closely migrating bands in polyacrylamide-SDS gels may not indicate two proteins of different size, but may indicate two proteins having different conformations in SDS. However, they also stated that it might be possible to separate two proteins of similar size on SDS gels.

Table 2—Variances within treatments[a] for selected parameters used to evaluate raw muscle and muscle heated to 25° or 45°C.

Parameter	Treatment				Significance
	Raw 1[b]	25°C	45°C	Raw 2[b]	
Cooking losses (%)					
Drip	–	0.07c	0.82d	–	**
Total	–	0.66c	2.70d	–	**
Number of heme bands stained with o-dianisidine	0.44c	3.25d	2.75d	0.50c	**

[a] Variances followed by the same letter are not significantly different at the 1% level of probability.
[b] Raw 1 and Raw 2 indicate treatments used as a reference point for muscle heated to 25° or 45°C, respectively.
** $P < 0.01$

In this study not all of the observed heme bands in stained gels were detected spectrophotometrically. Scans of gels after electrophoresis generally revealed five regularly appearing absorbance peaks of similar shape and location (Fig. 5). Occasionally, more absorbance peaks than the five measured could be detected; for example, the small peak preceding peak 1 in scan A. However, there was no regular pattern in appearance of the smaller peaks. Migration of peak 1 varied ($P < 0.05$) among treatments; an explanation for this is not readily apparent. Migration distances of other peaks measured did not vary significantly among treatments (Table 1).

Possibly heme pigments were not altered sufficiently at 25° or 45°C to result

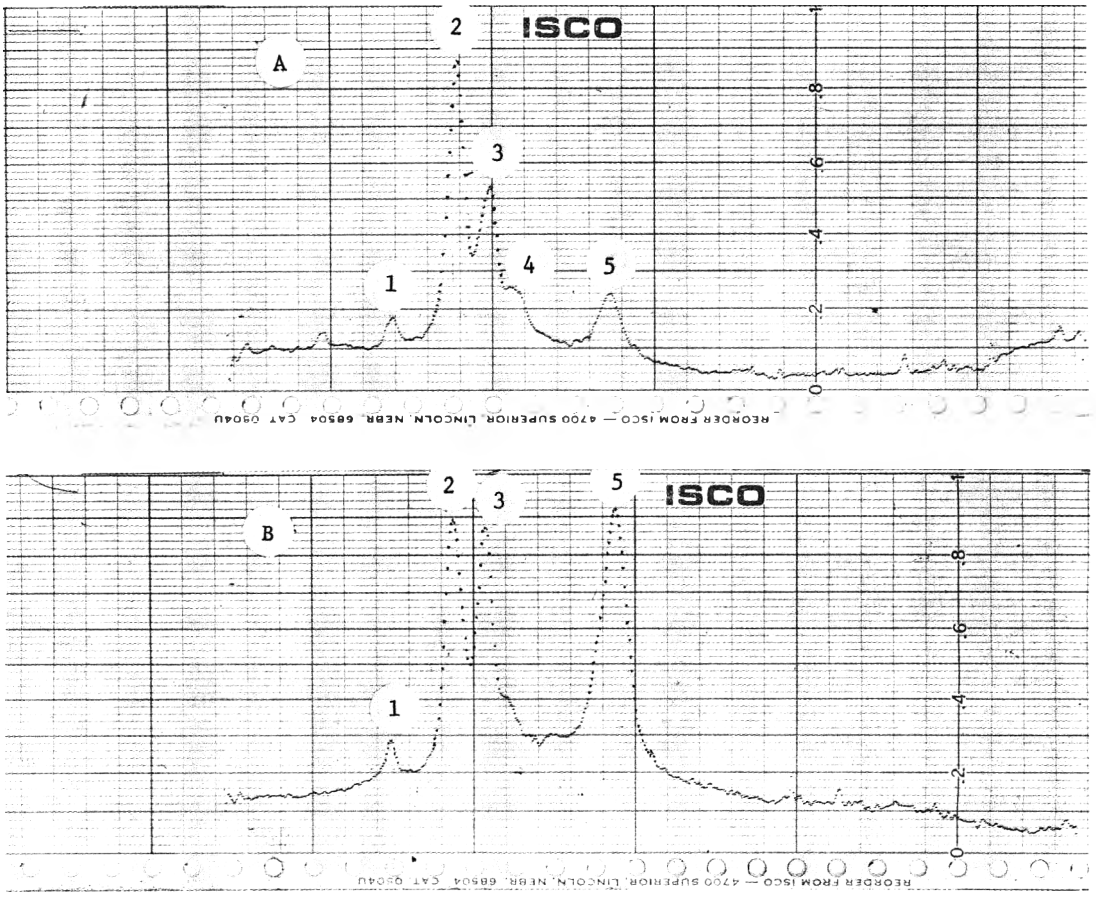

Fig. 5—Absorbance at 405 nm (0.2 absorbance = full scale) of typical unstained SDS-polyacrylamide gels. Gels were scanned with an ISCO photometric gel scanner in conjunction with a model UA-4 absorbance monitor and a type 5 optical unit. Scan of an electrophoretic gel containing sarcoplasmic extract from raw muscle shown in A and from heated muscle in B. Numbers on scans refer to absorbance peaks selected to measure migration.

in significant variation in migration of components investigated. Perhaps appearance of additional heme-positive proteins after heat treatment of muscle reflects alterations in size or composition of pigment molecules. Although ligand bonding to Mb reportedly is covalent and SDS reportedly disrupts hydrogen and hydrophobic bonds, the possibility that SDS interfered with detection of differences in migration distances of components investigated cannot be overlooked.

Of the peaks measured, the component designated peak 4 varied most in frequency of appearance. In scans of electrophoretic gels containing sarcoplasmic extracts from raw muscle, frequency of peak 4's appearance varied between samples from the two ends of the muscle. Heating had various effects of the appearance of peak 4. In scans of gels containing sarcoplasmic extract from muscle heated to an internal temperature of 25°C, peak 4's appearance decreased relative to its appearance in scans of gels containing sarcoplasmic extracts from the raw "control" (R 1). In scans of gels containing sarcoplasmic extract from muscle heated to 45°C, peak 4's appearance increased relative to that in scans of gels containing sarcoplasmic extracts from both raw "controls" and from muscle heated to 25°C. That pattern of appearance for component 4 could be attributable to alteration of the conformation and/or composition of heme pigments or heme pigment derivatives. Perhaps such changes in the sarcoplasmic proteins were in an intermediate stage at 25°C that did not allow extraction and detection of component 4. At 45°C, more or less permanent conformational and/or compositional changes in the component could have altered its migration through the gels, and allowed its detection.

Integrals were obtained to quantitate relative amounts of the five components measured (Table 1). Irrespective of internal end-point temperature, heating of muscle generally was associated with increased absorbance of the sarcoplasmic components. Only absorbance of peaks 1, 3 and 5 varied significantly among treatments (peaks 1 and 3, $P < 0.01$; peak 5, $P < 0.001$). The absorbance of those peaks always was significantly larger in scans of gels containing sarcoplasmic extracts obtained from muscle heated to 45°C than in scans of gels containing extracts from other treatments (Table 1). That may reflect an increase in the amount of heme pigment in the migration area or an increased absorption by those components at 45°C. Absorbance of peaks 1, 3 and 5 did not differ significantly among sarcoplasmic extracts from raw samples and muscle heated to 25°C. For all treatments, absorbance generally was smallest for peak 4 and always was largest for peak 2.

Burkhard and Stoltzenberg (1972) reporting on spectral changes caused by adding SDS to ferricytochrome c, a heme compound, postulated a two-phase interaction between those compounds. They noted that in the first phase SDS anions were bound to the cationic sites of the protein, a process which caused the protein to unfold and Soret absorbance to increase and shift to a shorter wavelength (408 nm). They further suggested that the protein might be unfolded completely in experimental systems that produce maximum hyperchromaticity in the Soret region and concomitant shifting of the Soret absorbance to 408 nm. They also postulated that the second phase of the interaction involved binding of the hydrophobic portions of SDS to the hydrophobic amino acid residues of the protein, and effected a partial loss of initial hyperchromaticity of the Soret peak with a further shifting to a shorter wavelength (406 nm). On the basis of the reported relationship between molecular weight and migration distance, peak 5 in this study could represent cytochrome c. Lehninger (1970) stated that cytochrome c is extracted easily from mitochondria by strong salt solutions. Although in this study a strong salt solution was not used to extract the sarcoplasmic fraction, Neelin and Ecobichon (1966) reported that intracellular muscle structures could be ruptured and the contents solubilized by extraction with hypotonic salt solutions.

Hyperchromaticity of absorbance peaks found in sarcoplasmic extracts of muscle heated to 45°C also could indicate an increase in absorbance in the Soret region similar to that reported by Awad and Deranleau (1968). They observed that Mb in relatively pure solution at pH 6.85 and above 80°C exhibited an increase in Soret abosrbance before precipitation. Keeping in mind the increased lability of Mb in muscle as compared to that in pure solution (Bernofsky et al., 1959; Draudt, 1969), it is possible that the increase in absorbance of components in this study is similar to that observed by Awad and Deranleau (1968). According to Ledward (1971) denaturation by heat is the only condition of denaturation under which such an increase in the Soret absorbance can be found for Mb. However, the specific effects of SDS on Mb were not considered as a denaturating condition.

Nelson (1972) stated that no studies had been reported on the effects of SDS on hydrophobic portions of molecules. The interior of Mb is hydrophobic in nature (Lehninger, 1970). Tung and Knight (1972) concluded that the unique primary structure of Mb may resist complete transformation by SDS into an unfolded, rod-like structure, usually attributed to the effects of SDS on proteins. In view of those reports, hyperchromaticity observed in this study possibly represented a unique effect of SDS on the heme environment of myoglobin.

Relationships among selected parameters

Correlation coefficients were calculated for each parameter measured vs every other parameter using pooled data for raw samples or data for each heat treatment. Significant ($P < 0.05$ or $P < 0.01$) coefficients that ranged from moderate to high ($r = 0.40$ to 0.70; $r = 0.80$ to 1.0) were studied. Heating was associated with a decrease in the number of significant correlations and generally it was associated with increased strength in correlation. In general, correlation coefficients showed expected relationships between parameters for intact heated muscle. Correlations between parameters of intact muscle and those of sarcoplasmic extracts, or those between parameters of sarcoplasmic extracts could not be explained.

CONCLUSION

THE COMPLEX NATURE of the sarcoplasmic fraction combined with the heterogeneity of bovine myoglobin make it difficult to relate color changes in muscle to changes in electrophoretic behavior of heme components. Under the conditions of this study, it was not possible to attribute spectral and electrophoretic pattern changes of components investigated to any single factor.

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COMPOSITION AND PROTEIN EFFICIENCY RATIO OF PARTIALLY DEFATTED CHOPPED BEEF AND OF PARTIALLY DEFATTED BEEF FATTY TISSUE AND COMBINATIONS WITH SELECTED PROTEINS

INTRODUCTION

PARTIALLY DEFATTED (PD) chopped beef and PD beef fatty tissue are products which according to USDA specifications, are produced in low temperature rendering of beef tissues particularly at a maximum temperature of 48.9°C.

Low temperature rendering originated because it is capable of producing high quality lard and tallow with improved color, odor and taste (Pavia, 1950; Swift and Hankins, 1952; Kramer, 1954a, b; Orsi, 1957; Downing, 1958, 1959; Sullivan, 1959; Christianson, 1961; Little and Milleville, 1963). This rendering is economically most feasible when profitable uses can be made of the resulting partially defatted tissues. One use was made possible as a result of USDA regulations which permit the use of limited amounts in meat products, i.e., 15% of the meat in frankfurters labeled "with by-products."

The products and by-products are designated according to species source and composition of the material rendered, PD chopped beef and pork being prepared from tissue containing at least 12% lean, classified as meat products, and PD fatty tissue from material containing none to an amount less than 12% lean, classified as by-products. (Currently, there is a proposal by USDA to amend its meat inspection regulations which includes reclassifying PD chopped beef and PD chopped pork as meat by-products.) The present study was concerned with products of bovine origin, PD beef fatty tissue, PD chopped beef and a third type, PD cured cooked chopped beef.

This study represents cooperative effort between the Animal & Plant Health Inspection Service and the Agricultural Research Service, USDA, to obtain analytical and nutritional data on samples of the above edible rendered tissues.

EXPERIMENTAL

TWO PD chopped beef, one PD cured cooked chopped beef, and three PD beef fatty tissue samples from six commercial establishments were analyzed for proximate and amino acid composition. Protein efficiency ratios (PER) of the partially defatted products were obtained. PER's were also obtained on lean beef, on a sample of collagen, and on combinations of lean beef protein with one each of a PD chopped beef or of two PD beef fatty tissue, and with combinations of whey or soy protein concentrates with one each of two PD beef fatty tissue samples.

Preparation of samples

Partially defatted products. The samples consisted of six boxes, 22.7 kg each, of partially defatted frozen products: three of beef fatty tissue, two of chopped beef, and one of cured, cooked chopped beef. They were stored in the range −23.3 to −20°C for a maximum of 2 wk. In preparation for freeze drying samples for feeding tests and analyses, each 22.7 kg frozen sample was cut into slabs with a band saw, ground in a Hobart meat grinder using a 1/2-in. plate opening and well mixed in a Buffalo ribbon blender for 5 min, using dry ice to keep the samples frozen. Aliquots of 1.5 kg and 4.5 kg of each ground frozen product were stored frozen until used for proximate analysis of the original materials and for preparation of freeze-dried samples, respectively.

Samples of each partially defatted product were freeze dried to less than 1% moisture in a Stokes shelf dryer at 41.6–43.3°C with a vacuum of 0.5 mm mercury. The dryer trays were each loaded with 2.3 kg of a ground frozen partially defatted product. 18 hr total drying time was necessary. During drying of the cured cooked chopped beef sample fat separated from the tissue and defatting was required before grinding. A 2.5-kg sample was washed successively with about 2 liters of Skellysolve F on Whatman #2 filter paper. The washed sample after drying weighed about 1.2 kg.

Each of the above products, after mixing with powdered dry ice, was ground as quickly as possible in a dry-ice cooled Wiley mill to pass a 2-mm screen. The ground samples were placed in loosely tied plastic food storage bags and refrigerated at 3–5°C until the carbon dioxide was vaporized. They were then well mixed and aliquots of each were removed for proximate and amino acid analyses and for feeding tests.

Lean beef. Six eye-of-round muscles (*semitendinosus*) were trimmed to remove outside fat, ground through a Hobart meat grinder using a 1/2-in. plate to prepare a 12 kg sample of lean beef, and well mixed in the Buffalo mixer. Because fairly large pieces of meat remained in the mixture, it was again ground in the Hobart, using a 3/16-in. plate, and again well mixed. The lot was sampled for proximate analyses by taking several aliquots of the ground beef, totaling 660g, combining and mixing them. The remainder of the sample was frozen in Stokes shelf drying trays, 1.8 kg of lean beef per tray, and dried in the Stokes dryer as described above in 12–14 hr. The dried samples were ground, stored, mixed and sampled as described above.

Collagen. The dried collagen used in this study was a sample removed from a large-scale lot prepared as follows: 200 lb of food-grade, limed cattlehide splits from a tannery were cut 1/4 in. wide by a Taylor-Stiles stripper and recut through a Taylor-Stiles granulator with 1-in. screen openings; the hide pH was adjusted to about 6 with lactic acid solution in a tanning drum, followed by water washing of the hide pieces. The pieces were then cut to 0.060 in. with the Urshel "Comitrol" and freeze dried as described for the partially defatted products. The dried samples were ground, stored, mixed and sampled as described above.

Chemical analyses

Official methods of the AOAC (1970) for meat and meat products were used to determine moisture, ash, fat (petroleum ether extractables) and Kjeldahl nitrogen. Percentage protein was calculated from the total nitrogen using the factor for the protein(s) analyzed, i.e., N X 6.25 for meat protein.

Amino acid analyses were determined in duplicate using the Piez and Morris system (1960) on samples that were extracted with petroleum ether to remove the fat, dried in a vacuum oven at 50°C, and then hydrolyzed with 6N HCl for 24 hr. Tyrptophan was determined on a separate sample hydrolyzed with methanesulfonic acid (Liu and Chang, 1971). Each amino acid in Table 3 was calculated as grams of amino acid residue per 100 grams of total amino acid residues.

Protein efficiency ratios

Protein efficiency ratios (PER) were determined at the USDA Western Regional Research Center, ARS, on the partially defatted products, lean beef, collagen, a whey protein concentrate (ENRPRO 50), a soy protein concentrate (PROMOSOY-100), and on several combinations of these proteins using the meth-

Table 1—Analyses of PD chopped beef, PD beef fatty tissue and lean beef

Product[a]		As received						Moisture free			
No.	Description	Moisture (%)	N (%)	Protein N X 6.25 (%)	Fat[b] (%)	Ash (%)	Total H_2O, prot, fat & ash (%)	N (%)	Protein N X 6.25 (%)	Fat[b] (%)	Ash (%)
5	PDCCCB	48.8	3.07	19.2	29.1	1.8	98.9	5.99	37.4	56.7	3.6
6	PDCB	71.4	2.91	18.2	9.5	0.9	100.0	10.15	63.4	33.2	3.3
2	PDCB	63.5	3.85	24.1	13.0	0.8	101.4	10.54	65.9	35.6	2.3
3	PDBFT	60.7	3.17	19.8	17.4	1.4	99.3	8.12	50.8	44.2	3.4
4	PDBFT	63.7	2.96	18.5	16.7	1.0	99.9	8.15	50.9	46.0	2.8
1	PDBFT	61.8	3.30	20.6	17.1	0.9	100.4	8.63	53.9	44.9	2.5
	LB	72.6	3.48	21.8	4.6	1.1	100.1	12.72	79.5	16.6	4.0

[a] Product code: PDCCCB, partially defatted cured cooked chopped beef; PDCB, partially defatted chopped beef; PDBFT, partially defatted beef fatty tissue; LB, lean beef
[b] Extracted with petroleum ether (30–60°C boiling temperature range)

Table 2—Analyses of PD chopped beef, PD beef fatty tissue, lean beef, and other protein sources

Product[a]		Freeze-dried products									
		As dried						Moisture free			
No.	Description	Moisture (%)	N (%)	Protein (%)	Fat[b] (%)	Ash (%)	Total H_2O, prot, fat, & ash (%)	N (%)	Protein (%)	Fat[b] (%)	Ash (%)
				N X 6.25					N X 6.25		
5	PDCCCB	9.0	12.34	77.1	6.5	7.7	100.3	13.56	84.8	7.1	8.5
6	PDCB	3.4	9.79	61.2	33.0	3.4	101.0	10.13	63.3	34.2	3.6
2	PDCB	4.0	9.93	62.1	33.7	2.2	102.0	10.34	64.6	35.1	2.3
3	PDBFT	3.2	8.05	50.3	43.8	3.0	100.3	8.32	52.0	45.2	3.1
4	PDBFT	6.8	7.72	48.3	43.0	2.6	100.7	8.28	51.8	46.1	2.8
1	PDBFT	3.6	8.24	51.5	43.7	2.4	101.2	8.55	53.4	45.3	2.5
	LB	5.0	11.94	74.6	16.1	4.0	99.7	12.56	78.5	17.0	4.2
				N X 5.62					N X 5.62		
	Collagen	6.6	15.20	85.4	5.0	2.0	99.0	16.28	91.5	5.4	2.2
Other proteins products											
				N X 6.38					N X 6.38		
	WPC[c]	6.7	8.15	52.0	0.8	10.1	69.6	8.72	55.6	0.9	10.8
				N X 6.25					N X 6.25		
	SPC	6.8	10.47	65.4	1.9	6.1	80.2	11.23	70.2	2.0	6.6

[a] Product code: PDCCCB, partially defatted cured cooked chopped beef; PDCB, partially defatted chopped beef; PDBFT, partially defatted beef fatty tissue; LB, lean beef; WPC, whey protein concentrate; SPC, soy protein concentrate.
[b] Extracted with petroleum ether (30 to 60°C boiling temperature range).
[c] Percent lactose was 21.5.

od of Derse (1965). The method involved feeding rats a diet containing 10% protein (N X 6.25, except N X 6.38 for whey and N X 5.32 for collagen), supplied by the test protein only, for 4 wk. Rats of the Sprague-Dawley strain (5 per group) were used. Casein was fed as the protein in the control diet for each PER Trial. The PER values were corrected to that of casein at 2.5. Standard deviation was determined and Duncan Multiple Range tests were calculated on the actual PER values before correction to 2.5.

RESULTS & DISCUSSION

BEEF fatty tissue ranges around 85–93% fat, 6–13% water and 2–3% protein. Fatty tissue with meat would contain considerably less fat and more protein and water, depending on the percent of meat present. During low-temperature rendering, the fat cellular tissue retains some fat and only relatively small quantities of water are evaporated. The resulting tissue residues contain appreciable quantities of fat and water, and proteins in the range 18.0–24.0%. The proteins are those of connective tissue, principally collagen, and those of muscle tissue, which include about 1% collagen, proteins of the actomyosin complex, and water-soluble proteins, such as myoglobin, enzymes and nucleoproteins.

Products as received

All partially defatted products appeared to be rope-like extrusions, about 1-1/2 in. in diameter, coiled into a corrugated box and hard frozen. The chopped beef products were light beige and pinkish in color. The beef fatty tissue products were pinkish to reddish brown in color.

Table 1 shows the proximate analyses

of the six partially defatted products and the lean beef sample. The fat content, approximately 17%, and the moisture content, 60.7–63.7% of the three PD beef fatty tissue products were fairly uniform. There was greater variation of fat and moisture in the PD chopped beef products. Generally, the moisture present was inversely proportional to the fat. When the analyses were calculated on a moisture-free basis (m.f.b.), each type product tended to have a distinctive compositional pattern.

Products after freeze drying

The dried and ground PD products had a ground-fiber appearance. The beef fatty tissues were mainly brown with a reddish tint with some light colored material intermixed. The chopped beef and cured cooked chopped beef were mainly beige intermixed with brown. Lean beef was uniformly light brown with a reddish tint.

Bacteriological tests, conducted to determine the wholesomeness of the materials, indicated that after freeze drying all products, lean beef and collagen were negative for coliforms and Salmonellae except one chopped beef product which had a count of 23 coliforms per gram.

The proximate analytical data are in Table 2. When the proximate data are calculated m.f.b., there is agreement between the nitrogen, fat and ash analyses of the products as received (Table 1) and after freezing drying (Table 2), excepting the cured cooked chopped beef. This product was extracted with Skellysolve F to lower the fat, which facilitated grinding and produced a more uniform sample.

Table 3 shows the amino acid composition grouped as essential and nonessential amino acids, and PER values for the proteins of each product, and of lean beef and collagen. Histidine is included in the list of essential amino acids because it is essential in the rat diet. Cystine and tyrosine also have been included with the essential amino acids because cystine can replace part of the methionine requirement and tyrosine part of the phenylalanine requirement (Rama Rao et al., 1961; Rose and Wixom, 1955a, b).

The data show that in these partially defatted products and collagen, the quantity of nonessential amino acids varies, and that this variation is largely accounted for by the differences in glycine, hydroxylysine and hydroxyproline. Collagen has the greatest amounts of these three nonessential amino acids (Table 3). Lean beef and the products with the highest totals of essential amino acids, one chopped beef and the cured cooked chopped beef, had the lowest quantities of glycine and little or no hydroxylysine and hydroxyproline, indicating that the collagen content was low in these products. The amounts of glycine, hydroxylysine and hydroxyproline in the other chopped beef and the three beef fatty tissue products indicate that the proteins of these products contained larger amounts of collagen.

Table 3–Amino acid analyses and PER values of PD beef products, lean beef and collagen (Grams of amino acid residue per 100 grams of total amino acid residues)[a]

		Partially defatted products						
		Chopped beef			Beef fatty tissue			
Amino acid	Lean beef	Cooked, cured 5	6	2	3	4	1	Collagen
		Essential amino acids						
His	3.6	2.9	3.0	2.1	1.8	1.9	1.9	0.8
Ile	5.0	5.2	4.1	3.2	2.9	2.7	2.6	1.6
Leu	8.3	7.8	7.2	6.1	5.8	5.7	5.3	3.0
Lys	8.8	8.6	7.9	5.3	5.3	5.2	4.9	3.7
Met	2.6	2.7	1.9	1.2	1.3	1.2	1.0	0.7
1/2 Cys	1.3	1.0	1.0	1.3	1.1	1.1	0.9	–
Phe	4.9	4.4	4.2	3.9	3.8	3.6	3.4	2.1
Tyr	3.9	3.7	3.1	2.3	2.3	2.2	2.0	0.9
Thr	4.4	4.2	3.8	2.7	3.0	3.2	2.7	1.9
Tryp	1.3	0.7	0.6	trace	trace	trace	trace	–
Val	5.5	4.6	4.6	5.8	4.8	4.9	4.6	2.3
Total	49.6	45.8	41.4	33.9	32.1	31.7	29.3	17.0
		Nonessential amino acids						
Ala	6.0	5.9	6.4	8.9	7.9	8.4	8.5	8.6
Arg	6.5	7.3	7.5	6.8	7.1	6.7	7.2	8.6
Asp	9.6	9.2	8.6	6.5	7.3	7.1	6.7	5.7
Glu	15.7	15.0	14.5	10.7	11.3	11.6	10.6	10.2
Gly	4.9	6.0	8.6	14.9	14.6	15.3	16.5	21.5
Hyl	–	0.7	1.0	0.9	0.8	0.9	1.1	1.3
Hyp	–	1.8	2.9	5.6	6.5	6.4	7.0	10.8
Pro	4.2	4.6	5.7	8.8	9.3	8.5	9.9	13.3
Ser	3.6	3.7	3.7	3.0	3.4	3.5	3.3	3.0
Total	50.5	54.2	58.9	66.1	68.2	68.4	70.8	83.0
Approx % of total N accounted for	81.9	88.5	82.7	85.8	83.6	84.0	85.6	92.8
PER value	2.85	2.58	2.38	1.61	1.70	1.68	1.13	<0

[a] Grams of amino acid residue per 100 grams of total amino acid residues may be converted to grams amino acid residue/100 grams of protein by multiplying by the % total nitrogen accounted for, and dividing by 100. For example, the value for histidine in lean beef becomes 2.95.

Although the sum of glycine, hydroxylysine and hydroxyproline varies among lean beef and the six products, the totals of the remaining nonessential amino acids remain rather constant, ranging from 44.7–46.4%. The total nonessential amino acids supply nitrogen for the synthesis in the body of any nonessential amino acids which may be lacking (Rose and Wixom, 1955c).

Results of the PER tests are shown in Table 4. Based on nitrogen digestibility, the range was 85–93% indicating that the observed nutritional value of the product proteins should be related directly to their amino acid composition. Such a relationship between the amino acid composition of each product analyzed and its PER value is apparent from the data. Examination of the PER values of these products and lean beef shows they are directly proportional and highly correlated ($r = 0.97$) to the total quantity of essential amino acids present. The proportion of each essential amino acid present to the total essential amino acids also can be expected to be reflected in the PER values. In fact, the correlation coefficient (r) of the linear regression ranges from 0.92–0.98 for the individual essential amino acids excepting valine. A correlation coefficient could not be calculated for tryptophan.

The data further indicate that the presence of small amounts, even traces, of tryptophan, the most limiting amino acid in partially defatted products, had a strikingly beneficial effect on the PER value.

Lean beef had the highest essential amino acid composition and the highest PER value, 2.85, significantly better than those of the six products. It is included as a reference meat protein and, as intended, supplied a parameter for interpreting the data. This amino acid analysis of lean beef is similar to that of Schweigert and Payne

Table 4—Rat PER data on PD beef products, lean beef, a whey protein concentrate, a soy protein concentrate, collagen, casein and combinations of these protein sources[a]

Dietary protein source[b,c]			Final mean body wt[e]	PER[f]	
No.	Description	% Nitrogen digestibility[d]	g ± std dev	Actual std dev[g] ±	Corrected
5	PDCCCB	92	182 ± 21	3.66 ± 0.25^{B}(1)[h]	2.58
6	PDCB	90	179 ± 15	3.38 ± 0.11^{B}(1)	2.38
2	PDCB	90	103 ± 4	2.28 ± 0.11^{C}(1)	1.61
3	PDBFT	85	117 ± 11	2.42 ± 0.17^{C}(1)	1.70
4	PDBFT	91	117 ± 13	2.39 ± 0.19^{C}(1)	1.68
1	PDBFT	88	84 ± 6	1.61 ± 0.19^{D}(1)	1.13
	LB	93	231 ± 15	4.05 ± 0.12^{A}(1)	2.85
	Collagen	88	43[i] ± 1	Wt loss (1)	–
	Casein	94	146 ± 15	3.55 ± 0.16^{B}(1)	2.50
	WPC	90	192 ± 23	3.84 ± 0.20^{A}(3)	2.74
	Casein	–	172 ± 13	3.51 ± 0.17^{B}(3)	2.50
	SPC	90	142 ± 21	3.05 ± 0.19^{CD}(5)	2.18
			Protein combinations		
	2/3 LB + 1/3 of #1	88	182 ± 20	3.24 ± 0.22^{CDE}(4)	2.40
	2/3 LB + 1/3 of #2	90	194 ± 34	3.39 ± 0.26^{A}(2)	2.46
	2/3 LB + 1/3 of #3	89	199 ± 20	3.50 ± 0.14^{A}(2)	2.54
	Casein	94	168 ± 15	3.45 ± 0.16^{A}(2)	2.50
	2/3 WPC + 1/3 of #1	86	162 ± 14	3.47 ± 0.24^{BC}(4)	2.57
	2/3 WPC + 1/3 of #3	88	187 ± 10	3.68 ± 0.08^{AB}(4)	2.72
	Casein	93	164 ± 24	3.38 ± 0.31^{BCD}(4)	2.50
	2/3 SPC + 1/3 of #1	86	141 ± 7	2.78 ± 0.09^{D}(5)	1.99
	2/3 SPC + 1/3 of #3	87	139 ± 11	2.79 ± 0.13^{D}(5)	1.99
	1/4 LB + 1/4 WPC + 1/4 SPC + 1/4 of #1	88	194 ± 24[j]	3.47 ± 0.14^{B}(5)	2.48
	1/4 LB + 1/4 WPC + 1/4 SPC + 1/4 of #3	89	219 ± 5	3.67 ± 0.06^{AB}(5)	2.62
	Casein	93	173 ± 19	3.50 ± 0.14^{B}(5)	2.50

[a] Rat feeding tests ran 27 or 28 days
[b] All diets contained 10% protein
[c] Product code: PDCCCB, partially defatted cured cooked chopped beef; PDCB, partially defatted chopped beef; PDBFT, partially defatted beef fatty tissue; LB, lean beef; WPC, whey protein concentrate; SPC, soy protein concentrate
[d] Nitrogen digestibility = N intake – fecal N/N intake X 100.
[e] Five weanling male rats per group. Initial age, 21 days; initial mean body weight, 54, 55, or 56g
[f] PER = protein efficiency ratio = weight gain (grams)/ protein intake (grams)
[g] Within each PER trial, means without a superscript letter in common are significantly different. Probability = <0.01.
[h] (1) = PER trial No.
[i] Data on three rats, one death
[j] Data on four rats

(1956). They found that the 18 amino acids assayed accounted for approximately 85% of the total nitrogen present in the meat.

There was no significant difference between the PER values of one chopped beef (2.38) and of the cured cooked chopped beef product (2.58) or between either one and the PER for casein (2.5). Chopped beef samples by definition or requirement are prepared from materials containing 12% or more separable lean; it can be presumed that the lean beef protein in the chopped beef samples reflects the percentage of protein from this source. One chopped beef, product 2, and two beef fatty tissue products had significantly lower PER values, 1.61, 1.70 and 1.68, respectively. There was no significant difference between these three. One beef fatty tissue had a low PER value, 1.13, significantly different from the values for all other products and lean beef. The results indicate that a statistically significant variation existed between beef fatty tissue products, undoubtedly relating back to variations in the stock material rendered.

PER values for combinations of proteins were: 2.40 for 2/3 of the protein supplied by lean beef and 1/3 by product 1, beef fatty tissue; 2.46 for 2/3 supplied by lean beef and 1/3 by product 2, chopped beef; and 2.54 for 2/3 supplied by lean beef and 1/3 by product 3, beef fatty tissue. These values were significantly greater than those of the three products separately, and were close to or the same as that of casein at 2.5.

The PER value was 2.57 when 2/3 of the protein was supplied by a whey protein concentrate and 1/3 by product 1, beef fatty tissue, and was 2.72 when 1/3 of the protein was supplied by product 3, another beef fatty tissue. These values were significantly greater than the value for either beef fatty tissue product separately.

PER values for combinations of two proteins, 2/3 from a soy protein concentrate and 1/3 from either product 1 or product 3, were 1.99. The data indicate that the proteins of lean beef or the whey concentrate supplement the PD beef fatty tissue proteins better than the proteins of the soy concentrate.

PER value for a combination of proteins, 1/4 from lean beef, 1/4 from whey protein concentrate, 1/4 from soy protein concentrate, and 1/4 from either product 1 or product 3 was the same or better than that of casein.

The U.S. Recommended Daily Allow-

ance of the protein in a food product is 45g if the PER of the total protein in the product is equal to, or greater than that of casein; and 65g if the PER of the total protein in the product is less than that of casein. Total protein in a food product with a PER less than 20% of the PER of casein is to be labeled "not a significant source of protein" (Federal Register, 1973). These considerations indicate that a partially defatted chopped beef with a PER of 2.38 would be nutritionally acceptable when used alone although is is not the equal of meat (PER 2.85). The other partially defatted chopped beef and the partially defatted beef fatty tissue products would be acceptable as food ingredients, provided they are blended with meat, whey protein concentrate, or vegetable protein foods, the amino acid composition of which supplements the amino acids of the partially defatted products.

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Reference to brand or firm name does not constitute endorsement by the U.S. Dept. of Agriculture over others of a similar nature not mentioned.

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PHYSICO-CHEMICAL CHARACTERIZATION OF NORMAL AND PSE PORCINE MUSCLE MYOGLOBINS

INTRODUCTION

PORCINE MYOGLOBIN (Mb) has been shown to be a very unstable molecule by Satterlee and Zachariah (1972) and Zachariah and Satterlee (1973). The myoglobin molecule from normal porcine muscle is more susceptible to heat and acid denaturation and to autoxidation at freezing temperatures, when compared to bovine and ovine myoglobin.

Myoglobin from muscle of stress susceptible pork has not been characterized to the same extent as other aspects of the pale soft and exudative (PSE) muscle obtained from stress susceptible animals. Briskey et al. (1959a, b, c) have shown that PSE muscle has a high initial glycogen level, a low ultimate pH, high expressible water ratios and high muscle sodium levels. Hart (1961) found the Mb concentration to be lower in PSE muscle, when compared to normal porcine muscle. Briskey and Wismer-Pedersen (1961) stated that PSE muscle does not have a significantly lower amount of myoglobin when compared to the normal muscle. Brown (1972) in a preliminary study found no significant differences in myoglobin concentrations between normal and PSE muscle, but did find that a Mb extract from PSE muscle autoxidized at a rate approximately double that of an extract from normal muscle.

The purpose of the present study was to characterize and compare in detail the properties and stabilities of extracts, crude Mb preparations and purified Mb from normal and PSE muscle.

EXPERIMENTAL

Isolation and purification

The porcine muscle used in the study was obtained through the courtesy of Iowa State University, the University of Wisconsin and a local meat packer. The PSE muscle was differentiated from normal muscle by visual observations. Those muscles with severe loss of color, moisture and being very soft were chosen to represent PSE muscle. Myoglobin from normal and PSE porcine muscle was isolated and purified according to the procedure of Satterlee and Zachariah (1972). Because of the greater instability of Mb from PSE muscle, the above procedure was modified by: (1) eliminating the dialysis and pervaporation procedures and (2) utilizing a DEAE cellulose column to concentrate as well as partially purify the myoglobin.

Autoxidation rates

Oxymyoglobins (MbO_2) were prepared according to the procedure of Brown and Mebine (1969). All autoxidation reactions were run in 10 mM phosphate buffer at pH 6.0 or in water, if a muscle extract was being tested.

Heat stability of MbO_2

The conversion of oxymyoglobin to metmyoglobin (MetMb) with increasing temperature was recorded spectrophotometrically by measuring the loss of 580 nm absorbance of the MbO_2 solution while it was heated to increase the temperature 0.8°C/min. Heating was performed in a water bath and the absorbance at 580 nm was measured at 5 degree intervals. All conversion rates were measured in pH 6.0, 10 mM phosphate buffer.

Infrared spectra

All Mb samples analyzed were freeze dried, then mixed with anhydrous KBr and formed into a micropellet. Infrared analyses were performed using a Beckman IR-12. To ensure that the sample after freeze drying was in the same form as it was prior to drying, a portion of the powder was rehydrated and analyzed via visible light spectroscopy.

Circular dichroism

The α-helical content was measured on the various purified myoglobin preparations using a Jasco ORD/CD-5 circular dichrograph. Each Mb sample to be analyzed was in a 10 mM, pH 7.0, phosphate buffer and was adjusted to an absorbance of 1.00 at 222 nm on a Cary 15 recording spectrophotometer. Spectra were recorded between 250 and 200 nm.

Other procedures

The isoelectric points (IP) of each of the Mb preparations were determined on an LKB Isoelectric Focusing apparatus according to the procedure of Satterlee and Snyder (1969).

Heat and acid denaturation curves were obtained according to the procedure of Satterlee et al. (1972).

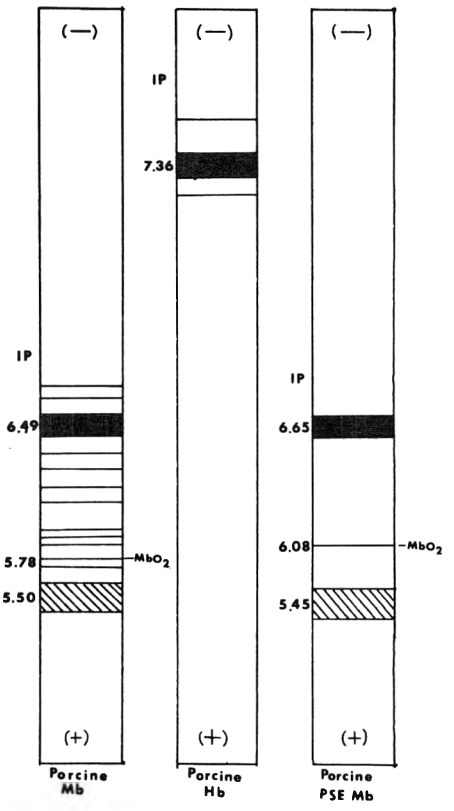

Fig. 1—Drawings of three isoelectric focusing columns showing patterns obtained from PSE Mb, normal porcine Mb and porcine hemoglobin (Hb). The ampholyte used was pH 6–8; a column temperature of 4°C was utilized.

RESULTS and DISCUSSION

DURING THE isolation and purification of PSE muscle myoglobin, it was noted that upon DEAE cellulose chromatography, a large green band was eluted just prior to the Mb band. This band was never seen upon purification of normal porcine Mb on a DEAE cellulose column and possibly represents a degradation product of porcine myoglobin.

The determination of the isoelectric points of normal porcine muscle Mb consistently shows the IP of MetMb to be 6.49 and MbO_2 to be 5.78. When PSE muscle myoglobin, either from the L. dorsi muscle or ham, was analyzed by isoelectric focusing, different isoelectric points were obtained for each muscle analyzed. Table 1 lists the IP for the met and oxy forms of normal and PSE muscle myoglobins. Metmyoglobins from PSE muscles varied from an IP of 6.29 to 6.69, while the IP of PSE MbO_2 varied from 5.58 to 6.09. This variance was not seen during the isoelectric focusing of normal porcine muscle Mb.

Upon isoelectric focusing of both normal and PSE porcine Mb, a large hemeprotein was noted which had a very low IP (4.5–5.5) and was much larger than any of the microheterogeneous Mb components.

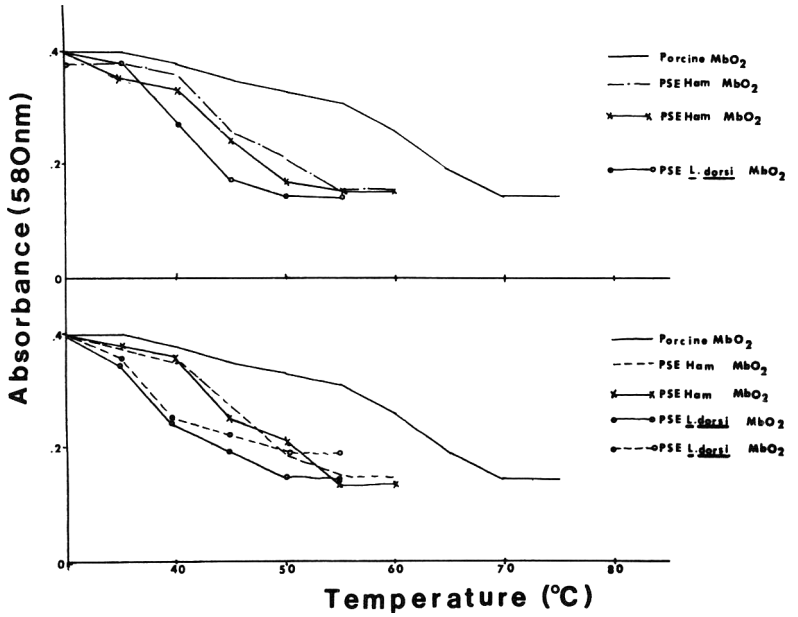

Fig. 2—Effect of increasing temperature on the conversion of MbO_2 to MetMb as measured by a loss of 580 nm absorbance. Performed in pH 6.0, 10 mM phosphate buffer.

Table 1—The isoelectric points (IP) for purified myoglobins extracted from various porcine muscles

Myoglobin	Isoelectric point	Avg dev from mean	No. of trials[a]
MetMb	6.49	0.01	8
MbO_2	5.78	0.01	6
PSE ham			
MetMb	6.66	0.00	2
MbO_2	6.09	0.01	2
PSE ham			
MetMb	6.56	–	1
MbO_2	6.08	–	1
PSE ham			
MetMb	6.52	–	1
MbO_2	5.80	–	1
PSE ham			
MetMb	6.60	–	1
MbO_2	5.78	–	1
PSE L. dorsi			
MetMb	6.59	–	1
PSE L. dorsi			
MetMb	6.61	0.01	2
MbO_2	6.09	0.01	2
PSE L. dorsi			
MetMb	6.34	–	1
MbO_2	5.58	–	1
PSE L. dorsi			
MetMb	6.29	–	1
MbO_2	5.70	–	1
Porcine MetHb	7.36	–	1

[a] Each trial represents a separate muscle extract for MetMb and MbO_2. Trials on the PSE samples represent single or duplicate analyses on an extract of that sample.

The low IP component was examined using molecular weight determination on Sephadex G-100 gel filtration chromatography and isoelectric focusing, and was not found to be porcine hemoglobin or a polymer of porcine myoglobin. It is approximately the same molecular weight as is normal porcine myoglobin. It acts as does a normal hemeprotein, in that it can exist in an oxidized (met) and a reduced oxygenated (oxy) state. The low IP component, present with both normal and PSE, is not believed to be a microheterogeneous Mb component, because of its high concentration. It is a Mb component unique only to porcine Mb.

Figure 1 illustrates the isoelectric focusing patterns of normal and PSE muscle MetMb and porcine MetHb.

The stabilities of the MbO_2 from normal and PSE muscle were compared by measuring the autoxidation rates of both muscle extracts and purified Mb at 5 and 30°C. Table 2 gives the autoxidation rate constants for normal and PSE muscle extracts and Mb. The autoxidation rates for PSE muscle extracts are much faster than that of a normal muscle extract. The autoxidation rate for the PSE L. dorsi extract was the fastest. When the MbO_2 fractions were purified, those from the PSE muscles were very unstable compared to MbO_2 from normal porcine muscle. At 5°C the rate constant differences are significant, and when determined at 30°C, these differences are even greater.

Another method to determine the stability of MbO_2 is to measure the rate

Table 2—Autoxidation rate constants of muscle extracts and purified oxymyoglobins obtained at both 5 and 30°C

Extracts	pH	Rate constant 5°C	Rate constant 30°C
Control	5.8[a]	0.003	0.025
	6.0[b]	0.003	0.025
PSE L. dorsi	5.6[a]	0.015	2.721
	6.0[b]	0.015	2.721
PSE ham	5.7[a]	0.005	0.058
	6.0[b]	0.003	0.073
Purified Mb			
Control		0.027	0.119
PSE ham		0.237	1.096
PSE ham		0.195	1.433
PSE ham		0.297	—[c]
PSE ham		0.286	—[c]
PSE L. dorsi		0.616	1.204
PSE L. dorsi		0.568	—[c]
PSE L. dorsi		0.920	—[c]

[a] Distilled water extracts at natural pH
[b] pH 6.0 phosphate buffer extracts
[c] Due to sample size, 30°C runs were not performed.

at which it is converted to MetMb during progressive heating. Figure 2 illustrates the more rapid conversion of MbO_2 from PSE muscles, when compared to normal muscle MbO_2.

The point at which one-half of the MbO_2 has been converted to MetMb is the T_{C50} point. Table 3 shows the T_{C50} values for the conversion of normal and PSE MbO_2 as well as the rate constants for the heat mediated reaction. The T_{C50} for normal porcine MbO_2 is over 10°C higher than the value of any PSE MbO_2. Also the rate of the reaction is 1.5 to 2 times faster for the PSE MbO_2, when compared to normal porcine MbO_2. The experiments measuring the autoxidation and heat conversion rates of PSE MbO_2 show that it is very unstable, when compared to normal porcine MbO_2.

Figure 3 shows the heat denaturation curves of normal and porcine PSE MetMb. In all cases, the control or normal Mb is much more heat stable when compared to the PSE MetMb. Although all of the PSE MetMb were unstable when heated, they varied in that instability. Table 4 gives the T_{D50} values, the point where 50% of the MetMb molecules have denatured for the myoglobin samples. The T_{D50} value for the denaturation of normal MetMb is almost 10°C higher than the value for the PSE MetMb.

Another method used to determine the stability of a protein molecule was to measure its ability to resist acid denaturation. Figure 4 shows the acid denaturation curves for normal and PSE MetMb. During the acid denaturation of PSE MetMb, the initial phase was more rapid than that of normal MetMb, with the final phase nearly identical for al MetMb.

The instability of PSE MbO_2 and MetMb indicates that it may differ structurally from normal porcine myoglobin.

Table 3—Heat conversion temperatures (T_{C50}) at which one-half of the oxymyoglobins have been converted to metmyoglobin in 10 mM phosphate buffer, pH 6.0 and rate of MbO_2 conversion

Sample	T_{C50} (C)	Rate of conversion (Δ 580 nm/°C)
Control	59.5	0.007
PSE ham	45.5	0.012
PSE ham	44.5	0.012
PSE ham	44.0	0.010
PSE ham	43.0	0.011
PSE L. dorsi	40.5	0.017
PSE L. dorsi	38.0	0.013
PSE L. dorsi	36.0	0.011

Table 4—Heat denaturation temperature (T_{D50}) at which one-half of the myoglobin molecules were denatured in 10 mM phosphate buffer, pH 6.0

Sample	T_{D50} in (C)
Control	78
PSE ham	67
PSE ham	69
PSE ham	66
PSE ham	67
PSE L. dorsi	58
PSE L. dorsi	54
PSE L. dorsi	58

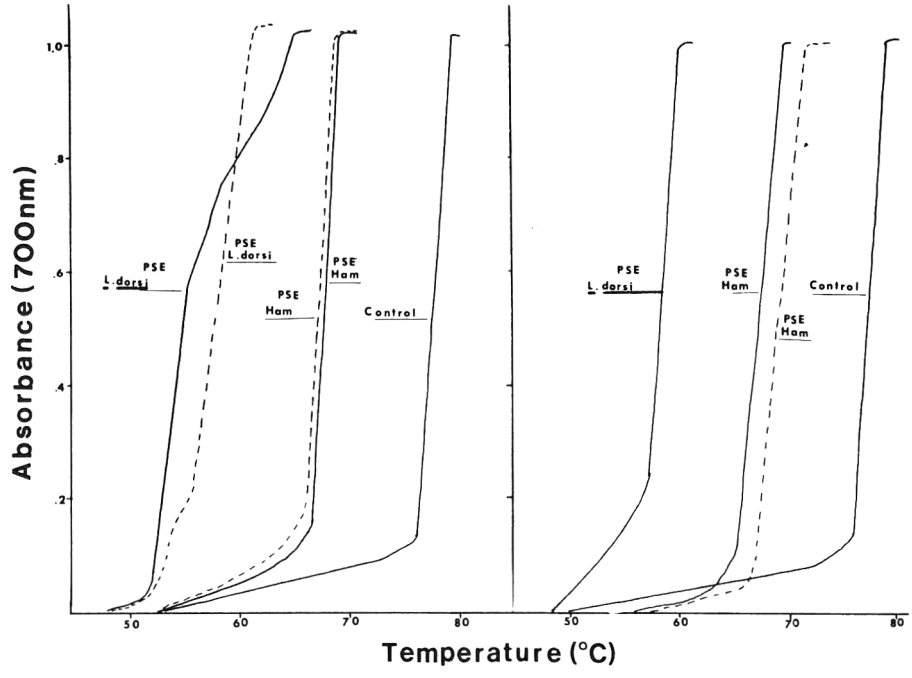

Fig. 3—Effect of temperature on heat denaturation of normal and PSE porcine metmyoglobin. Denaturation was detected by measuring the solution's light scattering at 700 nm. Performed in pH 6.0, 10 mM phosphate buffer.

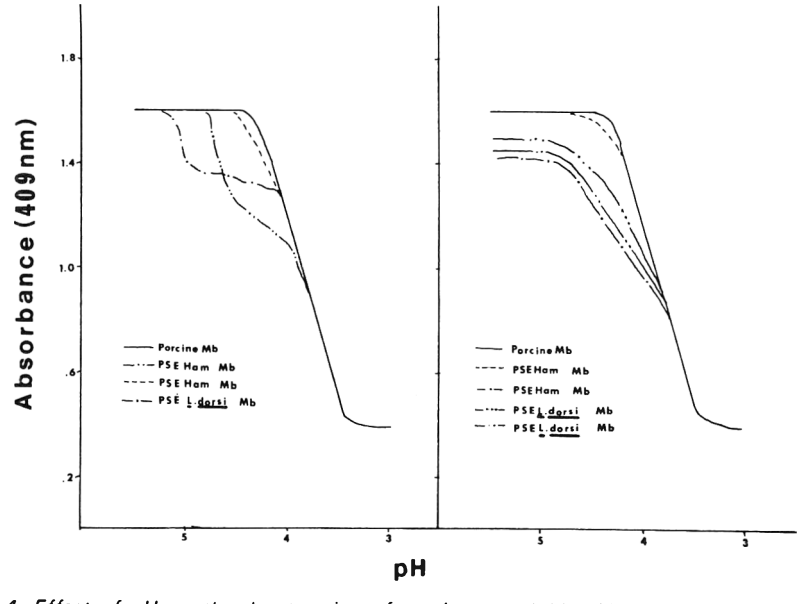

Fig. 4—Effect of pH on the denaturation of porcine myoglobin. All tests were performed in distilled water.

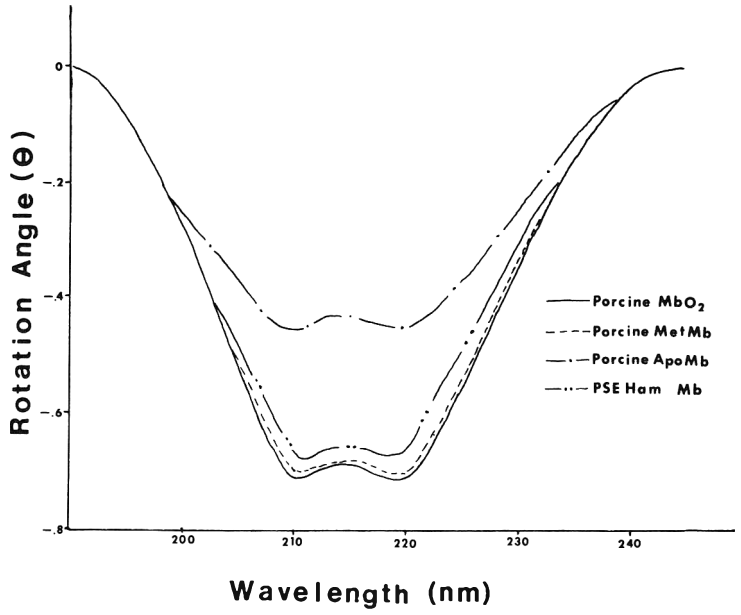

Fig. 5—Circular dichrograms of purified porcine myoglobins. All scans were run on Mb solutions in pH 7.0, 10 mM phosphate buffer having an absorbance of 1.00 at 222 nm.

Infrared spectral analyses indicated that no significant differences were present between normal and PSE MetMb. Figure 5 gives the CD spectra for the myoglobins. The measurement of the α-helical content of normal and PSE MbO_2 via circular dichroism (CD) indicated that the α-helix content of PSE MbO_2 is 4% lower than the α-helical content of normal porcine MbO_2.

CONCLUSION

THE INSTABILITY of myoglobin obtained from PSE muscle is first observed by denaturation during dialysis and purification on DEAE cellulose chromatography. This instability was further evidenced by the following:

1. Varying isoelectric points (IP), not one constant IP as seen with Mb from normal porcine muscle.
2. The larger autoxidation rate constants seen for extracts and purified Mb from PSE muscle, when compared to the same from normal porcine muscle.
3. Lower T_{D50} values for PSE muscle myoglobin which were lower by temperatures $\geqslant 9°C$.
4. The rapid conversion of PSE MbO_2 to MetMb by a factor 1.5 to 2 times faster than normal porcine Mb.
5. Denaturation of PSE Mb beginning at pH 5.1 to 4.5, when compared to normal Mb which begins to denature only when lowered to pH 4.3.
6. A lower α-helical content for the PSE MbO_2 molecule, when compared to normal porcine MbO_2.

The instability of PSE myoglobin could be a result of the physicochemical changes that occur within the muscle postmortem and this instability could contribute to the pale, soft, exudative condition of the porcine muscle. The rapid pH drop of PSE muscle, along with the low ultimate muscle pH could lead to partial denaturation of the myoglobin molecules. The instability of PSE MbO_2 to both heat and conversion to MetMb, could result in a rapid loss of the normal red muscle color and aid in the development of the pale color of PSE muscle. Denatured myoglobin will precipitate and settle out of the sarcoplasm, a factor known to be true for sarcoplasmic proteins in PSE muscle.

One question that arises from data presented in this study is why does the IP of PSE Mb vary, when normal porcine muscle Mb has a stable IP? A second question which also needs to be answered is, is the altered PSE Mb a result of genetic differences of PSE muscle or is it an artifact created by the stress occurring during death of a stress susceptible pork animal?

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Enterobacteriaceae AT VARIOUS STAGES OF POULTRY CHILLING

INTRODUCTION

THE EFFECTS of different methods of chilling poultry carcasses on microbiological quality, particularly the total bacterial count on the freshly processed poultry carcass, as well as the incidence and numbers of certain microorganisms of public health significance have been studied. Brewer et al. (1961), Kotula et al. (1962), Farrell and Barnes (1964), Surkiewicz et al. (1969), Keel and Parmelee (1968), Knoop et al. (1971), Veerkamp et al. (1972), Simonsen (1973) and Brant (1973) found that total bacterial counts on poultry carcasses were reduced during continuous immersion chilling. Thomson et al. (1965, 1966) reported no significant difference in the total bacterial count among poultry carcasses sampled at five locations in commercial immersion chillers. However, Clark and Lentz (1969), Lillard (1971) and Peric et al. (1971) reported an increase in total bacterial counts of carcasses during continuous immersion chilling.

Excessive numbers of *Enterobacteriaceae* may indicate greater food poisoning potential. Bulling and Pietzsch (1966) and Tamura et al. (1971) stated that the occurrence of *Salmonella* and enteropathogenic *Escherichia coli* in broilers is less likely if the *Enterobacteriaceae* count is low.

The objectives of this study were (a) to determine the changes in total plate count and the numbers and types of *Enterobacteriaceae* on carcasses chilled by continuous immersion or by a combination of immersion and air-blast chilling and (b) to determine numbers and types of *Enterobacteriaceae* on eviscerated carcasses during refrigerated storage.

EXPERIMENTAL

Locations of sampling

In-plant. Ten broiler carcasses were sampled at each of three locations in a commercial processing plant. Carcasses were sampled (1) before chilling, (2) after about 25 min in-line slush-ice immersion chilling and (3) after a combination of slush-ice followed by 45 min at −7°C air-blast chilling. Sampling was by swabbing a 12.3 cm^2 area of breast skin for 30 sec with a calcium alginate swab. Also water from the chiller was sampled for bacteriological analysis.

Storage. Ten freshly processed broiler carcasses were obtained from a local processing plant immediately after slush-ice immersion chilling. A 12.3 cm^2 area of skin on the left breast was swabbed and the *Enterobacteriaceae* count was determined. This was the storage day 0 sample. Each of the 10 birds was placed in a polyethylene bag and stored at 4°C until spoilage occurred (10 days). A 12.3 cm^2 area of skin on the right breast was swabbed to determine storage day 10 *Enterobacteriaceae* count. A preliminary study in our laboratory indicated that there was no significant difference in numbers or types of *Enterobacteriaceae* between the left and the right breast of poultry carcasses. *Enterobacteriaceae* cultures isolated from carcasses stored at 4°C for 10 days were tested for their ability to grow on brain heart infusion agar (Difco) at −2°, 1°, 4°, 25° and 35°C incubation for up to 21 days.

Microbiological methods

Total aerobic plate count with standard methods agar (BBL) was made for in-plant studies. Plates were incubated at 20°C for 72 hr.

To estimate the *Enterobacteriaceae* count, violet red bile agar (Difco) with 1% glucose was used (Mossel et al., 1962). The double-poured plates were incubated for 18–24 hr at 35°C. The counts were reported as logarithmic averages and expressed as microorganisms per cm^2 or per ml.

The experiment was replicated three times and for replications two and three of the in-plant study, and replication three of the storage study, *Enterobacteriaceae* were isolated from the highest dilution plated during microbiological analysis and identified to determine which genera of the *Enterobacteriaceae* predominated at each of the various chilling stages.

Three different systems were employed to identify *Enterobacteriaceae:* (1) the "R-B Enteric Differential System" manufactured by Diagnostics Research, Inc. is a screening device composed of a two-tube system which incorporates into one tube the tests for H_2S production, lysine decarboxylase, phenylalanine deaminase, lactose utilization and gas production from glucose, and in the other tube, the tests for indole production, ornithine decarboxylase and motility. (2) "Enterotube" manufactured by Roche Diagnostics is a screening device whereby 11 standard biochemical tests are inoculated simultaneously in the single compartmented tube from a primary plate; the Enterotube consists of media for testing citrate utilization, urease, phenylalanine deaminase, acid from dulcitol, acid from lactose, H_2S production, indole, ornithine decarboxylase, lysine decarboxylase, and acid and gas from dextrose. The ENCISE system, which is the *Enterobacteriaceae* numerical coding and identification system for Enterotube was utilized. (3) In addition to the above screening devices, further classification and identification of the *Enterobacteriaceae* were made according to the methods of Edwards and Ewing (1972).

RESULTS & DISCUSSION

In-plant study

Total plate count (TPC) and *Enterobacteriaceae* count (ENT) of carcasses sampled before chilling and after immersion and dry chilling declined significantly after chilling regardless of whether the birds were slush-ice chilled only or slush-ice plus air-blast chilled (Table 1). The average *Enterobacteriaceae* count after

Table 1—Total plate counts (TPC) and Enterobacteriaceae (ENT) counts of broiler carcass breast skin and chiller water during commercial chilling

Sampling location	Bacteria count[a]	
	TPC	ENT
Carcass before chill	3.17a	2.27a
Carcass after slush-ice chill	2.57b	1.48b
Carcass after combined slush-ice and air-blast chill	2.64b	1.02b
Slush-ice chiller water	3.26	2.87

[a] Carcass counts expressed as logarithms of number per cm^2; water counts per ml. Each value is the average of 30 samples. Means within a column followed by the same letter are not significantly different (P = 0.05).

Table 2—Genera of Enterobacteriaceae isolated from broiler carcass breast skin and chiller water during commercial chilling (average percentages—replications two and three only)

Locations sampled	Genus	%
Carcass before chill	*Escherichia*	90.7[a]
	Enterobacter	5.8
	Klebsiella	1.2
	Unclassified	2.3
Carcass after slush-ice chill	*Escherichia*	96.7
	Enterobacter	3.3
Carcass after slush-ice and air-blast chill	*Escherichia*	87.2
	Enterobacter	8.5
	Providencia	1.2
	Unclassified	2.1
Chiller Water	*Escherichia*	96.4
	Enterobacter	2.4
	Klebsiella	1.2

[a] The number of isolates for each location sampled ranged from 81–94.

slush-ice chilling was log 1.48/cm^2. This is in agreement with results reported by Mulder and Veerkamp (1973) who found that the *Enterobacteriaceae* count was reduced by one log after immersion chilling, when the immersion chilling was preceded by spray washing. A spray washer also preceded the in-line slush-ice chiller at the plant in which our study was made.

The *Enterobacteriaceae* in the chiller water and on the poultry carcass at the various stages of chilling (Table 2) were mainly of the genus *Escherichia* (87.2–96.7% of the isolates). Other genera found less frequently were *Enterobacter, Klebsiella* and *Providencia*. These findings agree with those of Berner et al. (1969) who also reported that *Escherichia* was the predominant genus of the *Enterobacteriaceae* isolated from carcasses immediately after chilling. The distribution of these species in the chill water and on carcasses at the various chilling stages were similar.

Salmonella, Shigella, Proteus and other genera of the *Enterobacteriaceae* group were not found. This does not indicate that they were not present either on the birds sampled or in the chiller water. If present in low numbers, it is unlikely that they would have been isolated from the higher dilutions. Secondly, their absence from the 12.3 cm^2 area of breast skin sampled does not preclude their presence elsewhere on the carcass.

Storage study

The number of *Enterobacteriaceae* on poultry carcasses stored for 10 days at 4°C increased significantly (Table 3). Carcasses were spoiled by this time and the *Enterobacteriaceae* count had increased by a factor of 10^2–10^3. To determine if the predominant genus or genera had changed during storage, the types of organisms isolated and identified from the samples on storage day 0 were compared with those from storage day 10 (Table 4). On storage day 0, *Escherichia* predominated, but after 10 days of storage, *Enterobacter* had become the predominant genus. These results agree with Berner et al. (1969) who reported that of the *Enterobacteriaceae*, the *Klebsiella-Enterobacter-Serratia* group predominated on spoiled poultry. All 98 *Enterobacteriaceae* cultures isolated from carcasses stored 10 days were identified as either *Escherichia* or *Enterobacter*, except one, *Klebsiella*. After incubation of the isolated cultures for 7 days at 1°C, none of the 14 *Escherichia* cultures grew, while 13 of the 83 *Enterobacter* cultures grew. After 21 days of incubation at 1°C, two *Escherichia* cultures had yet failed to show growth, while all 83 *Enterobacter* showed growth. At 4° and −2°C incubation, *Enterobacter* showed growth more frequently than *Escherichia*. All isolates were able to grow at 25° and 35°C incubation.

Our results indicate that immersion slush-ice continuous chilling, either alone or in combination with air-blast chilling, results in a significant reduction of both the total and *Enterobacteriaceae* count of broiler carcasses. The proportions of the different *Enterobacteriaceae* genera on carcasses chilled by the two procedures were similar. *Escherichia* was the predominant genus in both instances. Thus, air chilling to supplement immersion chilling of broiler carcasses appears to have little microbiological significance. Leistner et al. (1972) reported no difference in reduction in total and *Enterobacteriaceae* counts between a water spray chilling system and a combination water spray and air-blast system.

Enterobacter, which constituted only a small percentage of the *Enterobacteriaceae* on freshly processed carcasses, was the predominant genus of this family at the time of spoilage. *Enterobacter* isolates from the spoiled carcasses were shown to be more psychrophilic than *Escherichia*. Schultze and Olson (1960) reported that, in dairy products, psychrophilic strains of coliforms were more commonly found in the genus *Aerobacter (Enterobacter)* than in the genus *Escherichia*.

Additional information regarding the source, incidence and types of *Enterobacteriaceae* on poultry carcasses is needed to assess the significance of this group in poultry processing.

Table 3—Effect of 4°C storage on numbers of Enterobacteriaceae on broiler carcass breast skin

Storage time[a] (Days)	Enterobacteriaceae count
0	1.05[b]
10	3.63

[a] Birds had reached spoilage by the 10th day.
[b] Average of 30 counts (three replications); log of number per cm^2.

Table 4—Changes in percentage of each genus of Enterobacteriaceae on broiler carcass breast skin during 10 days of storage at 4°C

	Days stored	
Genus	0	10
Escherichia	85	14
Enterobacter	6	83
Citrobacter	7	–
Klebsiella	1	1
Providencia	1	–
Unclassified	–	2

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EFFECT OF GAMMA RADIATION ON CHICKEN LIVER CATHEPTIC ACTIVITY AND RELEASE OF LYSOSOMAL CATHEPSIN D

INTRODUCTION

MICROORGANISMS responsible for food spoilage can be effectively eliminated by sterilizing doses of radiation. However, residual enzyme activity may bring about undesirable changes as the radiation resistance of enzymes is much higher than that of microorganisms. Several studies have established the presence of intracellular proteolytic enzymes of lysosomal origin in skeletal muscle tissue which indicate that lysosomal cathepsins are an important group of enzymes in tissue systems as these are capable of intracellular protein breakdown (Bohley et al., 1971; de Duve, 1963; Reville et al., 1971).

The role of catheptic enzymes in postmortem proteolysis and meat tenderization is in dispute but there are several reports which suggest a release of catheptic enzymes and other lysosomal hydrolases during aging (Dutson and Lawrie, 1974; Eino and Stanley, 1973; Lutalo-Bosa, 1970; Ono, 1971). Gamma radiation has been demonstrated to result in differential release of acid phosphatase, β-glucuronidase and aryl sulfatase but have no effect on release of ribonuclease from lysosomes (Desai et al., 1964). Sottocassa et al. (1965), however, failed to find any effect of X-radiation on release of lysosomal β-glucuronidase or β-galactosidase. Large variation in radiation sensitivity of various enzymes and lysosomes from various sources is encountered in the literature. Moreover most of the studies have been done using rat liver tissue and no detailed results are available on cathepsins from poultry tissue. Therefore it was decided to study the effect of gamma radiation on the release of lysosomal cathepsins. Because postmortem skeletal muscle tissue has a lower catheptic activity as compared to organ tissue (Bailey and Rhodes, 1964; Musch et al., 1969) chicken liver was used as the experimental material and as the source of lysosomal fractions.

EXPERIMENTAL

COMMERCIAL BROILERS (8–12 wk old) were obtained from the University Poultry Farm and maintained under standard husbandry conditions for about a week prior to slaughter. The birds were sacrificed by exsanguination after fasting for 40–48 hr. The livers were rapidly removed and washed free of blood with cold 0.25M sucrose solution and chilled in crushed ice. All the subsequent operations were carried out at 4°C unless otherwise indicated.

Isolation of lysosomes

Excessive fat and connective tissue were removed from the livers of three to four birds. The tissue was minced with scissors and then mixed thoroughly. Lysosomes were isolated by sucrose density gradient and differential centrifugation techniques as described by Sawant et al. (1964b). The lysosomal pellet was suspended in 0.7M sucrose solution and 1:2 (w/v) dilution was made on the basis of liver tissue used for isolation of lysosomes. This final suspension was irradiated as described below.

Irradiation

The samples were subjected to varying doses of gamma radiation in a Gamma Cell-220 (Atomic Energy of Canada Ltd.). During radiation the samples were kept at low temperature by packing them in crushed ice which was charged periodically when necessary. The chicken liver tissue slices were subjected to radiation doses of 0, 0.05, 0.10, 0.25, 0.50 or 1.00 Mrads. The lysosomal suspensions received radiation doses of 0, 0.1, 0.25, 0.50 or 1.00 Mrads. The dose rate at the time of irradiation was 0.71 Mrads/hr.

Preparation of samples for enzyme study

Chicken liver tissue. The chicken liver tissue samples were homogenized in distilled water 1:20 (w/v) immediately after irradiation using a Sorvall Omnimixer. The homogenization was done at high speed for four 15-sec intervals with a 30-sec cooling period between successive runs. During homogenization the mixer container was kept in crushed ice. The tissue homogenate was centrifuged at 20,200 × G for 30 min in a Sorvall RC2-B refrigerated centrifuge. The supernatant was used for determination of enzyme activity designated as free catheptic activity. Another portion of the homogenate was subjected to freeze-thaw treatment ten times. Freezing was done by dipping into liquid nitrogen and thawing was done at 37°C in a water bath. Samples were then centrifuged as described above and this second supernatant was used to determine total catheptic activity. Total activity of irradiated samples was expressed as percent of control value and called residual enzyme activity.

Lysosomes. After irradiation the release of catheptic enzymes from lysosomal suspensions was followed at two different temperatures. One portion of the suspension was incubated at 37°C in a constant temperature water bath and samples were drawn after 0, 15, 30, 60, 120 and 180 min. The other portion of lysosomal suspension was kept at 4°C and samples were drawn after 0, 24, 48 and 72 hr. To determine free catheptic activity as a measure of released enzyme, the samples were centrifuged at 17000 × G for 20 min and catheptic activity of the supernatant measured. For determination of total catheptic activity of lysosomes, samples were given freeze-thaw treatment ten times and then centrifuged at 17000 × G for 20 min and the supernatant used for determination of total catheptic activity. Total catheptic activity remaining after irradiation treatment was expressed as percentage of control value and called residual activity.

Assay of catheptic enzymes

Cathepsin D activity of tissue and of lysosomal preparations was determined according to the method of Anson (1938) with some modifications (Berman, 1967).

The reaction mixture contained 1 ml of supernatant and 2 ml of 2% hemoglobin (Bovine, Type II–Sigma Chemical Co.) in 0.2M acetate buffer, pH 3.8. The reaction was carried out at 37°C for 2 hr in a constant temperature water bath with shaking and the reaction was terminated by addition of 2 ml of 10% TCA. The samples were kept overnight at 4°C and then filtered through Whatman No. 4 filter paper. The blanks were also prepared in a similar manner but were kept at 4°C instead of incubating at 37°C. The absorbance of the filtrate was read against respective blanks at 280 nm on a Unicam SP 800 B, UV spectrophotometer (Pye-Unicam Ltd.). The increase in absorbance was expressed as enzyme activity. All measurements were performed in duplicate.

Light scattering properties of lysosomes

After irradiation the lysosomal suspensions were incubated at 37°C in a constant temperature water bath. The absorbance of the suspensions was read at 540 nm after 0, 15, 30, 60, 120 and 180 min of incubation using a Spectronic 20 spectrophotometer (Bausch & Lomb). The instrument was set at zero by using irradiated 0.7M sucrose solution. The decrease in absorbance was used as an index of release of lysosomal enzymes (Sawant et al., 1964b).

RESULTS & DISCUSSION

IN OUR STUDIES on liver tissue, the free activity was significantly ($P \leq 0.05$) increased due to irradiation treatment (Tables 1 and 2), but the magnitude of the increase was smaller at higher doses. These results are in agreement with those

Table 1—Effect of low doses of gamma radiation on free catheptic activity of chicken liver tissue

Dose (Mrads)	Activity (%)[a]
Control	100.0a
50	118.7b
100	122.2b

[a] Means with different letters are significantly different ($P < 0.05$).

Table 2—Effect of high doses of gamma radiation on free catheptic activity of chicken liver tissue

Dose (Mrads)	Activity (%)[a]
Control	100.0a
0.25	113.2b
0.50	112.4b
1.00	111.9b

[a] Means with different letters are significantly different ($P < 0.05$).

of Coehlo (1969) who noticed an increase in ATPase activity at lower doses with progressive inhibition at higher doses of gamma radiation. Musch et al. (1969) reported no such increase in catheptic activity of fish muscle, but noticed that catheptic enzymes were highly radiation resistant. Doty and Wachter (1955) also failed to detect any change in proteolytic activity of irradiated beef muscle after 500,000 rep of gamma radiation but Klein and Altmann (1972) reported increased proteolytic activity after irradiation of chicken muscle.

In the present study it was found that total catheptic activity of liver tissue was considerably decreased ($P \leqslant 0.01$) at higher doses of radiation (Fig. 1). This does not preclude the possibility of radiation enhancing the release of catheptic enzymes resulting in increased availability. Isolated chicken liver lysosomal fraction was used for further studies, to permit monitoring of the release of enzymes in the suspension medium.

Light scattering has been used as an index of change in the shape of particles and release of enzymes (Sawant et al., 1964b). In our studies the application of gamma radiation resulted in a significant ($P \leqslant 0.01$) change in absorbance at 540 nm (Fig. 2). A-540 was significantly ($P \leqslant 0.05$) decreased by radiation doses up to 0.5 Mrad at all the time intervals while in 1.00 Mrad samples no significant decrease was observed after 60 min incubation. Decrease in A-540 is indicative of release of lysosomal enzymes. Lysosomes contain a variety of hydrolytic enzymes and release of any of these enzymes might occur to a varying degree. As we were mainly interested in cathepsins, free catheptic activity of lysosomes was monitored and is shown in Figure 3. The decrease in absorbance of lysosomal suspension was closely paralleled by an increase in free catheptic activity. An aggregation of particles in samples treated with 1.00 Mrad was noticed after 120 min incubation at 37°C and may be responsible for high absorbance at 540 nm (Fig. 2) and low free enzyme activity (Fig. 3).

The results of an experiment conducted to assess the effect of incubation of lysosomal suspensions at 4°C are shown in Figure 4. All of the irradiated samples showed significant increases in free catheptic activity ($P \leqslant 0.01$) compared to the control but the release of the enzyme was slower at 4°C than at 37°C incubation. Most probably slow release of cathepsin D at 4°C is due to radiation aftereffects such as free radical interactions, formation of secondary radicals and the resultant changes in the system which would proceed at slower rate at 4°C (Copeland et al., 1968). The pronounced increase in free enzyme activity at both temperatures suggests that irradiation does enhance the release of enzyme from lysosomes.

As shown in Figure 5, total catheptic activity of lysosomes was decreased by radiation treatment indicating that while irradiation might depress the total enzyme activity, it substantially increases free enzyme content (Fig. 4). Desai et al. (1964) found a decrease in absorbance at 540 nm of a lysosomal suspension and release of lysosomal enzymes into the suspension medium from rat liver lysosomes due to radiation damage. Similar findings were reported by Harris (1966, 1967) on noncatheptic enzymes in isolated lysosomes from rat liver. Results of the present study show general agreement with the foregoing reports.

The observed increase in free catheptic activity from lysosomal particles is most probably due to release of bound enzymes and leakage of enzymes due to rupture of lysosomes, or changes in permeability of lysosomal membrane. The

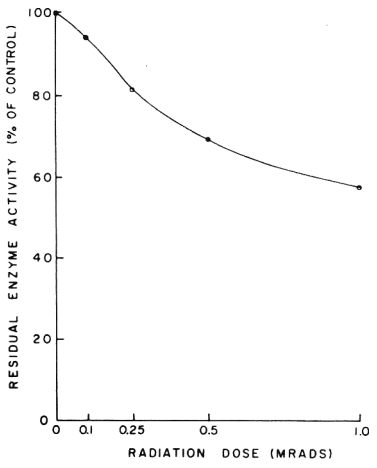

Fig. 1—Radiation inactivation of chicken liver tissue cathepsin D. Chicken liver tissue slices were irradiated at 0° C and total catheptic activity measured immediately after irradiation. Total activity of irradiated samples is expressed as residual activity given in percentage of control value.

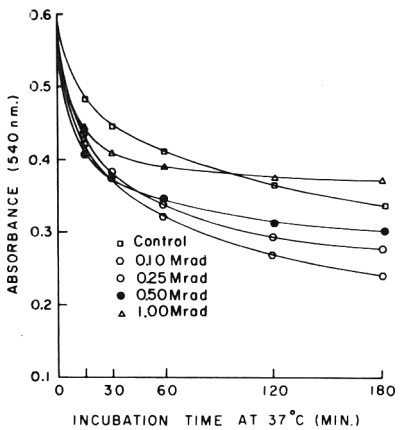

Fig. 2—Changes in light scattering properties of lysosomal suspensions after exposure to gamma radiation and subsequent incubation at 37° C. The lysosomal suspensions were irradiated in 0.7M sucrose, 0.001M EDTA solution, pH 7.0 at 0° C.

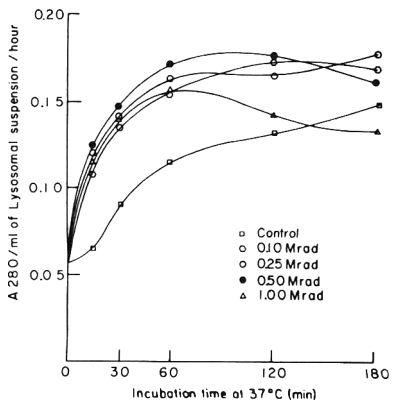

Fig. 3—Release of cathepsin D from chicken liver lysosomal fraction. Lysosomal suspensions were irradiated in 0.7M sucrose, 0.001M EDTA solution, pH 7.0, at 0° C and subsequently kept at 37° C.

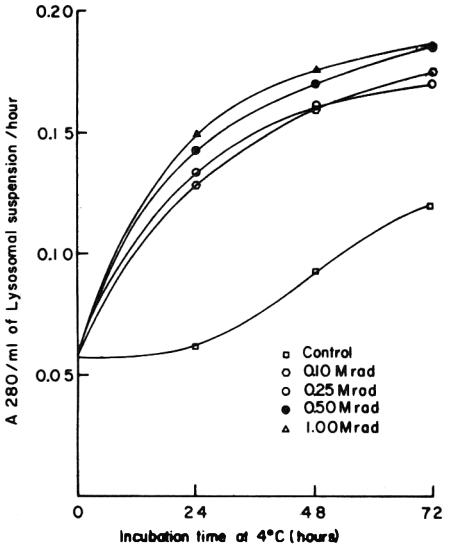

Fig. 4–Release of cathepsin D from chicken liver lysosomal fraction. Lysosomal suspensions were irradiated as described under Figure 3 and kept at 4° C after irradiation.

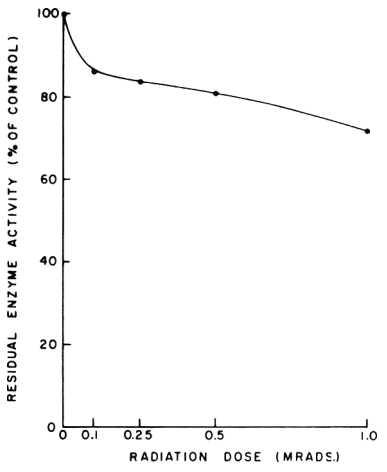

Fig. 5–Radiation inactivation of cathepsin D of chicken liver lysosomal suspensions. Lysosomal suspensions were irradiated as described under

radiation induced oxidation of sulfhydryl groups in intracellular membranes could damage their structure and might result in disruption of lysosomes (Sutherland and Pihl, 1968). Free radical formation by ionizing radiation has been suggested as an important factor contributing to the disruption of lysosomes (Desai et al., 1964).

There are reports suggesting that membrane bound cathepsins might be released by irradiation and partially digest the lysosomal membrane resulting in enzyme leakage due to change in permeability of lysosomal membrane (Beaufay and de Duve, 1959; Sawant et al., 1964a). Involvement of cathepsins in membrane permeability changes does not seem to be a likely cause of increased enzyme activity in the present studies as release of enzyme is very rapid immediately after irradiation (Fig. 3) and according to this hypothesis there should be slow release of enzyme initially, with a subsequent rise in free enzyme content after prolonged incubation.

Present studies indicate that radiation does affect the stability of lysosomes, possibly damaging membrane structure resulting in enhanced release of lysosomal enzymes. It requires further investigations to determine the definitive mechanism of such an action.

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EFFECT OF VACUUM PACKAGING ON MICROORGANISMS ON CUT-UP CHICKENS AND IN CHICKEN PRODUCTS

INTRODUCTION

SEVERAL HUNDRED different species of microorganisms have been reported on poultry meat. Ayres et al. (1950) reported that immediately after processing, chromogenic bacteria represented 50–60% of the total microflora on the carcass; *Pseudomonas*, colorless cocci and closely related forms represented 20–25%, while the remaining 20–25% of the microorganisms consisted of miscellaneous bacteria. When the cut-up poultry parts were stored until spoiled, chromogens and other miscellaneous bacteria accounted for less than 1% of the total microorganisms while *Pseudomonas* and *Alcaligenes* were found to be the principle microflora on slimy spoiled parts. It was stated in a later study by Ayres (1960) that *Pseudomonas* was the most significant gram negative nonspore forming rod associated with spoilage of poultry meat. On a study of the spoilage microflora of eviscerated chicken stored at different temperatures, Barnes and Thornley (1966) reported that the pseudomonads became less significant as holding temperature increased. According to their data, however, percent incidence of the members of the family *Enterobacteriaceae* increased from 3 at 1°C to 15 and 27 at 10 and 15°C, accordingly.

The effect of packaging materials and techniques on shelf life of fresh poultry meat has been reported by Wells et al. (1958). They showed that packaging materials, vinylidene chloride copolymer (VCP) and cellophane film did not exert a direct bacteriostatic effect on the spoilage microorganisms which developed in poultry meat. It was also reported by these researchers that shelf life for birds wrapped in cellophane and VCP were the same under nonvacuum conditions, but when the air was evacuated, the shelf life increased 4 days beyond that obtained without vacuum. It was also reported, but not explained, that the type of film and packaging may influence the type of off-odor which persists at the time of spoilage. Debevere and Voets (1973) indicated that bacterial growth at 4°C was inhibited by using a shrinkable polyvinyl chloride film with a permeability to oxygen of approximately 500 ml/m^2/24 hr under 1 atm. However, no attempt was made in that study to determine the anaerobic bacteria.

Shank and Landquist (1963) used various packaging films for storing meat products both vacuum and nonvacuum packed. They found that nonvacuum packaged meat spoiled faster than the vacuum packaged products. They observed also that most of the spoilage was due to lactic acid producing bacteria in vacuum packed meat, while in nonvacuum packed meat, large numbers of yeasts and molds were found. On a study of the microflora of irradiated petrale sole fillets packed in C-enameled cans and stored at 0.5°C, Pelroy and Eklund (1966) reported that the nonirridated vacuum packed fish spoiled after 7 days of storage. They also indicated that 94% of the microbial population at the time of spoilage was found to be the nonpigmented *Pseudomonas*. Little or no literature was available concerning the effect of vacuum packaging on the microbial quality of polyethylene film-packaged chicken products.

EXPERIMENTAL

Sample preparation

Commercially dressed broilers were obtained directly from a processing plant. Each carcass was cut-up into eight parts by cutting each half into breast, thigh, drumstick and wing portion. The cut-up parts were mixed to insure proper distribution of the microbial population. Only breast and thigh portions were randomly assigned to three packaging variables. One cut-up part was then placed in each bag. The packaging variables were: (1) vacuum packaged samples: 1.5 mil polyethylene pouches (All-Vak #13, International Kenfield Distributing Co., Broadview, Ill.) with 25 psi guage vacuum developed and heat sealed using a Kenfield Vacuum Packaging Sealer (International Kenfield Distributing Co.); (2) sealed samples: 1.5 mil polyethylene pouches heat sealed without vacuum; and (3) control samples: 1.5 mil polyethylene bags with the top of the pouches folded over without heat sealing. Eight replications were made from each packaging variable. The samples were placed in a 2–4°C refrigerator immediately after packaging.

Total count

Total counts were made at zero time, and were repeated every 4 days for a period of 28 days. Bacteriological samples of the raw broiler parts from the three packaging variables were obtained by swabbing the skin for 30 sec with cotton swabs (Falcon Plastic, Los Angeles, Calif.). An area of 1 in.^{2} or 6.45 sq cm described by a sterile aluminum foil template, was assayed. The sample swab was inserted about 2 in. into a dilution tube containing 10 ml of nutrient broth diluent and the cotton tip was broken off into the tube. The content of this serial dilution tube was shaken vigorously to disperse the cotton and to thoroughly mix the microflora. Plate counts were made employing the following media in duplicate: (1) Plant count agar (Difco); (2) Brewer anaerobic agar (Difco); and (3) Staphylococcus-110 agar (Difco). Plates were incubated for 72 hr at 20°C to determine the aerobic and anaerobic psychrophile counts, and 48 hr at 37°C to determine the mesophile counts. Total count on Staphylococcus-110 medium was made after 72 hr of incubation at 37°C. The average number of colonies from the duplicate plates was reported as the number of bacteria per sq cm of skin surface. The studies were repeated four times.

Isolation and characterization of spoilage microorganisms

After the samples had been stored for 28 days at 2–4°C, aerobic and anaerobic psychrophilic petri plates of these samples containing 10–30 colonies each were selected. The colonies were isolated and purified using the streak-plate technique.

The purified cultures were transferred onto nutrient agar slants and incubated for 24 hr at 25°C. Fresh cultures were used for the identification. Gram reactions and other selected tests as shown in Table 1 were conducted on each isolate according to the procedures described by Collins and Lyne (1970).

Selected *Enterobacteriaceae* sp. in vacuum packaged poultry products

Fresh cut-up broilers were cooked for 15 min in boiling water, hand deboned and ground using a Sears Kenmore meat grinder plate with 2.4 mm diam holes. The ground meat was autoclaved for 15 min at 15 psi steam pressure and 121°C. The sterilized meat was then sorted into 10-g portions and the portions were transferred into 1.5 mil polyethylene bags aseptically.

Fresh liquid cultures of *Aerobacter aerogenes*, *Escherichia coli* and *Proteus mirabilis* were prepared by inoculating one loopful of young cultures into nutrient broth and incubating at 25°C for 24 hr. 1 ml of the fresh broth cultures was inoculated into polyethylene pouches containing 10g of sterile meat. 25 psi guage vacuum was developed and the bags were heat sealed.

Contents were transferred aseptically into 90 ml nutrient broth at zero, 1, 2 and 3 wk of refrigerated storage at 2–4°C. Total counts were made as described earlier. Studies were repeated and results were reported as numbers of bacteria per gram of samples.

RESULTS & DISCUSSION

Effect of packaging on the microflora of cut-up broiler parts

The three packaging variables used did not affect the psychrophilic aerobic total counts or the shelf life of the cut-up fresh broilers (Fig. 1a). All parts spoiled almost at the same time. These results are in contrast with those of Spencer et al. (1956) and Debevere and Voets (1973) who reported that evacuating the air during packaging of fresh poultry meat tends to inhibit bacterial growth and increase the shelf life. After storage for 12 days at 2–4°C, distinct differences were recorded in the anaerobic psychrophile count of portions packaged in vacuum (Fig. 1b). It was also observed that the vacuum packaged samples had the highest anaerobic psychrophile counts throughout the entire refrigerated storage period (Fig. 1b). According to Elliott and Michner (1961) who indicated that off-odors and slime of poultry meat occurred when the log number of bacteria reached 7.5–9.0 per sq cm, our control samples were actually spoiled at 10–12 days of storage at 2–4°C. The anaerobic counts of control samples, however, were always below the

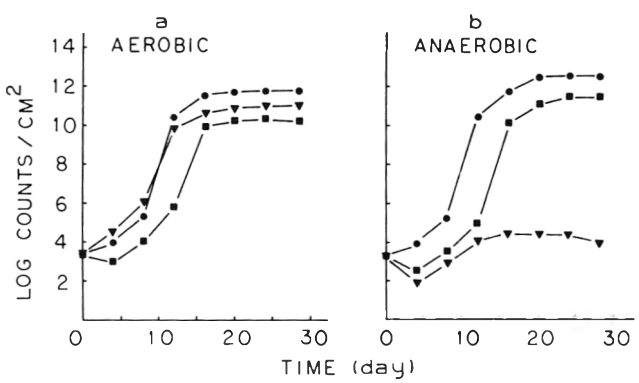

Fig. 1—Aerobic and anaerobic psychrophile counts of cut-up raw poultry stored at 2–4°C. ●, Vacuum packaged; ■, Regular sealed; and ▲, Control packaged.

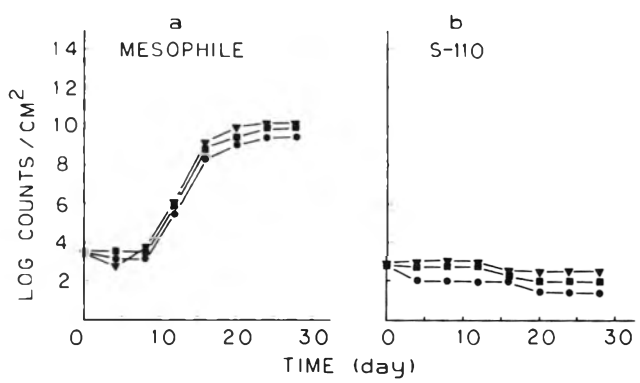

Fig. 2—Mesophilic and staphylococcus-110 medium counts of cut-up raw poultry meat stored at 2–4°C. ●, Vacuum packaged; ■, Regular sealed; and ▲, Control packaged.

Table 1—Summary of some physiological and biochemical characteristics of the spoilage microflora isolated from packaged poultry meat[a]

Source	No. of isolates	% of isolates	Gram stain	Motility 0.5% Agar	Motility Hanging drop	Lactose fermentation	Hugh and Leifson	H_2S	Cytochrome oxidase
Vacuum packaged parts	45	95.9	G- short rod	+	+	AG	Ferm.	–	–
	2	4.1	G- short rod	+	+	–	Ox.	–	+
Control packaged parts	59	95.8	G- short rod	+	+	–	Ox.	–	+
	3	4.2	G- short rod	+	+	AG	Ferm.	–	–

Source	No. of Isolates	% of Isolates	Catalase	Urease	MR	VP	Citrate	Indole	PA deamination test	KCN	Possible genus
Vacuum packaged parts	47	95.9	+	–	–	+	+	–	–	+	*Enterobacter*
	2	4.1	+	–	–	–	+	–	–	+	*Pseudomonas*
Control packaged parts	59	95.8	+	–	–	–	+	–	–	–	*Pseudomonas*
	3	4.2	+	–	–	+	+	–	–	+	*Enterobacter*

[a] AG, acid and gas: Ferm., fermentative: Ox., oxidative

spoilage borderline during the entire storage period. The 1.5 mil polyethylene film used in this experiment has a relatively low oxygen permeability (1.0 cc/100 in^2/24 hr at 25°C, 760 mm difference in pressure). When the 25 psi guage vacuum was developed, the film enveloped the parts tightly so that very little or only a trace amount of oxygen remained in the packet. The microorganisms responsible for spoilage of vacuum packaged samples were expected to be either anaerobe or facultative anaerobe. Control samples were not sealed leaving oxygen available for the aerobic bacteria to grow and dominate. Spencer et al. (1956) and Wells et al. (1958) noticed that the type of packaging treatment may influence the type of off-odors which persist at the time of spoilage, but no further explanation was given.

The total aerobic and anaerobic counts of the regular sealed samples were practically the same for the first 12 days. This observation can be explained on the basis that this type of packaged samples contained a limited amountg of oxygen that will allow the growth of aerobic bacteria. After all the oxygen in the bags was consumed, the condition then resembled that of the vacuum packaged samples and the anaerobic or facultative bacteria gained ascendance.

Staphylococcus medium-110 counts of cut-up fresh broilers decreased slightly for all packaging variables upon refrigerated storage (Fig. 2b). Vacuum packaged samples had the lowest counts as compared to those of the sealed and the control samples. No considerable variation was observed on mesophilic counts from the three packaging variables (Fig. 2a). Again, vacuum packaged samples had the lowest counts.

Results obtained from the above experiments showed clearly that vacuum packaging did not confer any protection of fresh poultry meat upon refrigerated storage. Borgstrom (1968) reported that vacuum packaging of meat will change the microflora, but will not inhibit bacterial growth. He stated further that vacuum packaging is not a preservation method, merely a tool.

Microorganisms responsible for spoilage of packaged raw broiler parts

Physiological and biochemical tests suggested that the bacteria isolated from vacuum packaged and control samples were different (Table 1). About 95.9% of the microorganisms found in vacuum packaged parts upon spoilage were members of the genus *Enterobacter*, while only 4.1% were found to be members of the genus *Pseudomonas*. In control packaged parts, however, members of the genus *Pseudomonas* were the dominant bacteria (95.8%) responsible for spoilage and only 4.2% were found to be *Enterobacter* species. For the vacuum packaged samples, an additional 49 colonies were isolated and purified from the Brewer anaerobic agar. Physiological tests indicated that they were the same type microorganisms as those isolated from plate count agar. It was evident from this study that *Enterobacter* sp. which represents a facultative anaerobe, will grow rapidly under vacuum conditions, outnumber other microflora, and cause spoilage of the vacuum packaged raw broiler parts under refrigerated storage.

It was also observed that different types of spoilage odors existed in the vacuum and the control packaged samples. This result agreed with Spencer et al. (1956) and Wells et al. (1958). The difference in the spoilage microflora as described previously might be responsible for this phenomenum.

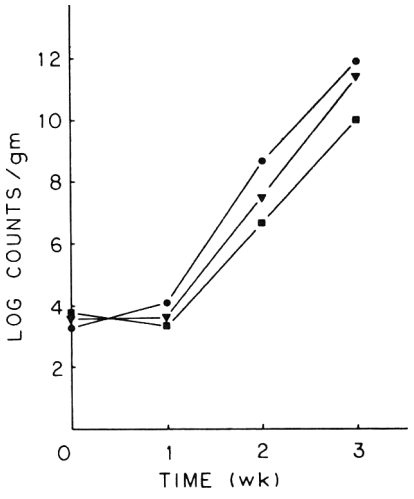

Fig. 3–Survival and growth of some species of the family Enterobacteriaceae on vacuum packaged cooked poultry meat stored at 2–4°C. ●, A. aerogenes; ■, E. coli; and ▲, P. mirabilis.

Growth of certain *Enterobacteriaceae* in vacuum packaged poultry products

Since the results obtained in this study indicated that members of the *Enterobacteriaceae* family grew in vacuum packaged fresh poultry, further work was done to investigate the possible growth of certain members of the family in the vacuum packaged poultry products.

It was found that *A. aerogenes, E. coli* and *P. mirabilis* could survive and grow in vacuum packaged, sterilized poultry meat upon refrigerated storage (Fig. 3). Many species of *Enterobacteriaceae* are primarily environmental saprophytes and scavengers, some of them are well known as pathogens. Results obtained in this study indicated that the potential survival and growth of these *Enterobacteriaceae* members in vacuum packaged food products should not be overlooked.

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CORRELATION BETWEEN THE FRESHNESS OF ICED SALMONIDS AND LACTIC ACID FORMATION IN MUSCLE EXTRACTS

INTRODUCTION

THE POSTMORTEM chemical changes that take place in fish flesh held at temperature above freezing are known to be due to (1) microbial activity, (2) nonenzymic catalytic processes such as autoxidation of lipids and (3) activity of muscle enzymes. The microbial contamination is of major importance in bringing about the deterioration of the quality of fish stores without freezing (Shewan, 1961; Liston, 1965) and several chemical tests have been developed to measure the bacterial spoilage of fish (Kreuzer, 1971). The autoxidation of highly unsaturated fish lipids is a problem encountered during the storage of various fishery products (Olcott, 1962; Labuza, 1971). In the case of fatty species kept in ice the storage life may be limited by the development of oxidative rancidity which sets in prior to bacterial spoilage (Hansen, 1972). By contract to microbial activity and autoxidative processes, the changes associated with the activity of muscle enzymes during ice storage generally do not result in the unacceptability of fish for human consumption. Nevertheless, since the autolytic changes begin immediately after death they may serve to assess the degree of freshness, i.e., prespoilage quality of fish. Among the indices of freshness based on changes in concentration of muscle metabolites, the determination of the degradation products of adenine nucleotides was shown to have the greatest value (for review see Gould and Peters, 1971). Attempts were also made to use the level of muscle enzyme activity as a criterion of freshness of iced fish (Shibata et al., 1969; Yamanaka and Mackie, 1971; Manohar and Boese, 1971; Masic and Hamm, 1971; and ref. cited by Gould and Peters, 1971).

The purpose of this study was to determine whether there is a correlation between the formation of lactic acid by the soluble muscle enzymes and postmortem age of iced fish. The fish used were rainbow trout (*Salmo gairdneri*) and sockeye salmon *(Oncorhynchus nerka)* stored in melting ice for 0–14 days. The test consisted in measuring the conversion of fructose-1,6-diphosphate (FDP) to lactice acid in muscle extracts, in presence and absence of added nucleotides.

MATERIALS & METHODS

Fish

Rainbow trout (*Salmo gairdneri*) were obtained from Sun Valley Trout Farm, Mission, B.C., and maintained under conditions described earlier (Bilinski et al., 1971).

Sockeye salmon (*Oncorhynchus nerka*) were caught in June 1973, when they entered Great Central Lake on Vancouver Island, B.C. The methods of capture and the conditions used for keeping salmon in captivity have been described by MacBride (1967). Within 4–6 wk after their arrival at the aquarium, the salmon were used in the cold storage experiments.

Storage and sampling of iced fish

Fish were killed by a blow on the head, gutted, washed with cold tap water and beheaded by a transverse cut at the base of pectoral fins. At this stage, "0 time," muscle samples were taken and fish were placed in cracked ice in boxes, stored at 5°C. Subsequently muscle samples were removed for enzyme assays after 3, 7, 10 and 14 days of ice-storage. Unless otherwise stated, only the dorsal anterior regions of the light (fast) muscle were sampled; the successive samples (5g) were obtained from the same fish starting at the anterior end. The surface layer (3–5 mm) of the transverse cut was discarded at each sampling. The dark lateral-line muscle and the fat-rich tissue adjacent to the skin were also carefully avoided. The posterior region of the dorsal muscle (referred to in Fig. 2) represents the tissue adjacent to the adipose fin.

Determination of lactic acid formation in muscle extracts

All operations, involving tissue preparation, were carried out at 3–5°C. A 1-g portion of muscle sample, cut into small pieces with scissors, was placed in a test tube (18 X 150 mm) containing 9.0 ml of Na_2HPO_4-KH_2PO_4 buffer, 100 mM, pH 7.4. The tube was maintained in an ice-bath, while the tissue was homogenized for 2 min (4 X 0.5 min) with a motor-driven Teflon pestle rotating at 1500 rpm. The homogenate was centrifuged at 1000 X G for 10 min and the supernate was decanted. Aliquots of the supernate were used for the enzyme assay and for determination of the lactic acid content before incubation (preformed lactic acid).

The standard assay mixture (3 ml) used for the determination of lactic acid formation had the following composition: 1.0 ml supernate (10 mg total protein); FDP, 5 mM; Na_2HPO_4-KH_2PO_4 buffer, 30 mM, pH 7.4; KCl, 50 mM; $MgCl_2$, 4 mM; nicotinamide, 40 mM (MacLeod

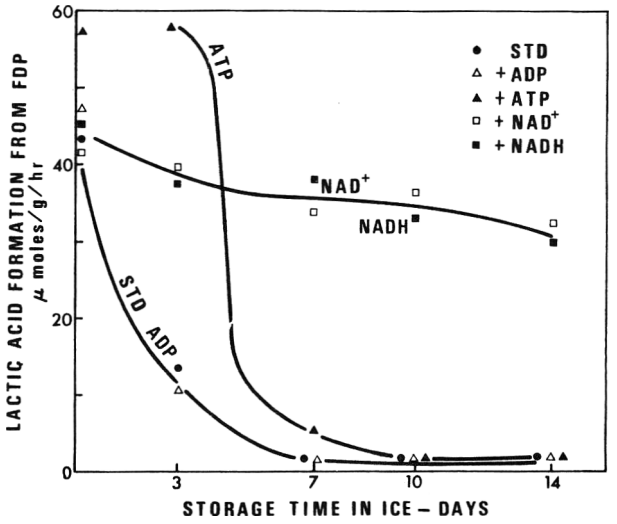

Fig. 1–Effect of storage time in ice on lactic acid formation from FDP in trout muscle. Assays were conducted under the following conditions: (●) standard assay (without added nucleotides); (△) + ADP, 1 mM; (▲) + ADP, 1 mM; (□) + NAD+, 0.3 mM; (■) + NADH, 0.3 mM. Values given in the figure represent averages for three fish.

et al., 1960). The final concentration of the nucleotides used in some of the assays was as follows: ADP, 1 mM; ATP, 1 mM; NAD^+, 0.3 mM; NADH, 0.3 mM. The incubation was conducted at 25°C in a constant-temperature bath provided with a shaking device, using 20 ml test tubes. Immediately before starting the incubation, tubes were flushed with N_2 and stoppered. At the end of the incubation (usually 120 min) 1.0 ml 8% perchloric acid was added to stop the reaction. After centrifugation at 600 X G for 5 min, aliquots of the supernate (0.2 ml) were used for the determination of lactic acid (= total lactic acid).

The level of enzymic activity in the muscle is indicated by the formation of lactic acid during the incubation, which amounts to the difference between the total and preformed lactic acid. All results are reported in μmoles lactic acid per g muscle (wet tissue) per hr. Lactic acid determinations were carried out according to Marbach and Weil (1967) by an ultraviolet method, involving the use of lactate dehydrogenase. Protein was measured by the Biuret method of Gornall et al. (1949).

RESULTS & DISCUSSION

Effects of assay conditions and other factors on lactic acid formation in muscle extracts

Rainbow trout was used in the experiments concerned with the development of the method. Preliminary tests indicated that in contrast to free glucose, the hexose phosphates (glucose-6-phosphate, fructose-6-phosphate and FDP) were readily converted to lactic acid by fresh trout muscle preparations. It was also found that this enzymic activity showed a significant decrease after storing fish in ice for 3 days. In subsequent experiments the conversion of FDP to lactic acid served to measure the postmortem changes in enzymic activity of the muscle. In preliminary tests it was established that the formation of lactic acid reached its maximum rate at approximately 5 mM concentration of FDP and showed a linear increase during the first 2 hr of incubation at 25°C. The effects from addition of nucleotides to the assay medium were determined in muscle stored in ice for 0–14 days (Fig. 1). In absence of added nucleotides the ability of the muscle to form lactic acid from FDP was greatly depressed already after 3 days of storage. The addition of ADP had no noticeable effect on these changes. When the assays were conducted in medium fortified with ATP, the enzyme activity remained at levels comparable to the fresh muscle up to 3 days of storage and decreased thereafter. In presence of added NAD^+ or NADH, the ability of the muscle to form lactic acid was maintained at comparatively high levels during 14 days of storage and there was no well marked difference in the effects from the oxidized or reduced form of the nucleotide. Although in the freshly killed fish there was no difference in the level of activity between the posterior and anterior regions of the dorsal muscle, the decrease in activity during storage took place at a faster rate in the latter (Fig. 2A and 2B). The two muscle regions also differed in their content of preformed lactic acid (Fig. 2D). The preformed lactic acid represents the amount of this compound present in the muscle at the time of death as well as that formed postmortem from carbohydrate stores of the muscle. In view of these differences it appeared essential to use samples from the same defined region of the muscle for the purpose of obtaining strictly comparative data. As shown in Table 1 there was little variation in the activity when several samples of the same fish were taken from different sections of the muscle located between the head and the dorsal fin (anterior region). It was also found that the rate of lactic acid formation was independent of the size of the fish and under the test conditions strenuous muscular effort prior to killing had no effect on the level of activity in the fresh muscle or on the rate of postmortem changes (Table 1). In additional experiments, not shown here, it was found that lactic acid formation was significantly depressed when the muscle sample was homogenized under drastic conditions such as using an omnimixer instead of a Teflon pestle.

In the foregoing experiments, the level of enzymic activity was determined by measuring the ability of muscle preparations to form lactic acid from an exogenous substrate. This approach differs from that used in earlier investigations on postmortem glycolytic changes in fish concerned with the degradation of carbohydrate stores of the muscle (Tomlinson and Geiger, 1962; Partmann, 1965; Fraser et al., 1967; Manohar, 1970, among others). The depletion of muscle glycogen during storage of fish was found to be associated with an increase in muscle lactate, although a stoichiometric relation was not always observed. In fish held at temperatures above freezing these changes are usually completed by the time muscle is in rigor and they are known to be affected by the premortem condition of fish (degree of exercise and stress, nutritional state). As far as FDP is concerned, it was shown in studies with the Atlantic cod, *Gadus callarias*, that this sugar phosphate occurs in the fresh muscle, but disappears within 2 days of storage of fish in ice (Burt, 1961, 1971; Burt and Jones, 1961). There is now abundant evidence that the formation of lactic acid in fish muscle takes place through the same sequence of enzymic reactions that are operating in mammalian muscle (Tarr, 1972). The metabolism of lactic acid is known to differ to some extent between the white and the red skeletal muscle of fish, as the latter is better adapted to carry on the oxidative processes (Bilinski and Jonas, 1972; Bilinski, 1974). The differences between the anterior and posterior regions of the white muscle observed in the present study confirm the earlier observations of Black et al. (1962), who found that lactic acid occurs at higher concentrations in the tail portion of trout muscle. The apparent inability of the

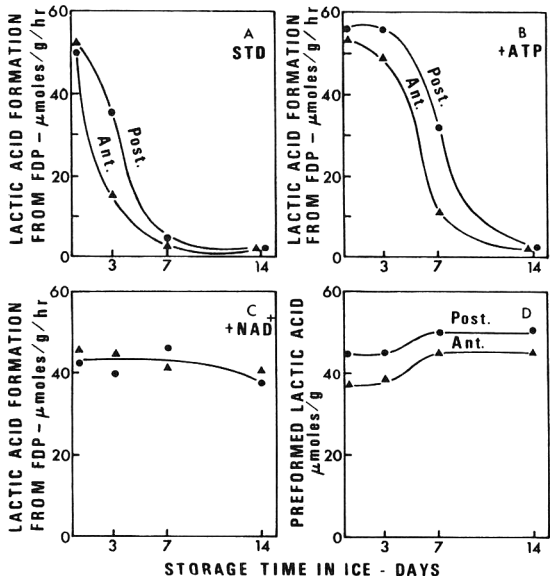

Fig. 2—Lactic acid formation from FDP and preformed lactic acid in the anterior (ant.) and posterior (post.) regions of trout muscle. For assay conditions see Figure 1.

white muscle of trout to metabolize free glucose has already been reported by MacLeod et al. (1963), who attribute it to low hexokinase activity in this tissue.

Our results showing the effects of the addition of nucleotides on lactic acid formation from FDP point to the conclusion that the observed decrease in glycolytic activity during ice storage of fish mainly reflects a progressive destruction of essential cofactors rather than an inactivation of enzymes. This is indicated by the fact that even after 2 wk of ice storage the glycolytic activity was largely restored by supplementing the assay medium with NAD^+ or NADH. In this respect it is interesting to note that in a study of glycolysis in minced mammalian muscle, it was shown that due to loss of NAD^+, the glyceraldehyde-3-phosphate dehydrogenase step became rate limiting for lactate production and FDP accumulated (Newbold and Scopes, 1971a). The addition of NAD^+ prevents this occurrence (Newbold and Scopes, 1971b). It was unexpected to find that in contrast to ADP, the addition of ATP led to a significant increase in glycolytic activity following ice-storage of trout. This stimulation must be ascribed to a secondary effect, since ATP is not required in the enzyme reactions responsible for the conversion of FDP to lactic acid. It is noteworthy that the mechanism by which ATP serves to restore the operation of glycolysis in the muscle extracts is progressively destroyed during the first week of ice storage. It may be pointed out that it is not possible to conclude whether our data are an accurate representation of postmortem changes in the intact muscle, as they could be to some extent affected by such factors as the partition of glycolytic enzymes between the sediment and the soluble phase of the homogenate (Melnick and Hultin, 1970).

The postmortem breakdown of fish muscle nucleotides during cold storage

Table 1—Lactic acid formation from FDP in the anterior region of trout muscle

	Lactic acid formation (μmoles/g/hr)			
	Duration of storage in ice (days)			
	0		3	
Fish	Standard assay	Assay with ATP	Standard assay	Assay with ATP
Multiple samples from the same fish				
Fish no. 1	50.84 ± 1.06(4)[a]	–	17.31 ± 0.73(4)	–
Fish no. 2	45.01 ± 0.77(4)	–	12.83 ± 0.57(4)	–
Fish no. 3	47.67 ± 1.24(4)	–	10.55 ± 0.43(4)	–
Total weight of fish				
100–150g	53.95 ± 6.30(10)[b]	–	–	–
300–400g	50.25 ± 4.77(6)	–	–	–
Degree of exercise before killing				
Free swimming	51.18 ± 6.94(6)[b]	52.83 ± 5.87(6)	10.59 ± 2.63(6)	44.90 ± 6.40(6)
Exercised[c]	50.37 ± 8.38(5)	54.04 ± 5.75(5)	11.39 ± 4.38(5)	50.54 ± 10.11(5)

[a] Mean value, standard deviation, and number of samples
[b] Mean value, standard deviation, and number of fish tested
[c] Before killing fish were chased for 15 min.

Table 2—Effects of storage time in ice on lactic acid formation from FDP in sockeye salmon muscle

Fish				Lactic acid formation (μmoles/g/hr)									
				Duration of storage on ice (days)									
				0	3	7	10	14	0	3	7	10	14
No.	Total wt (g)	Sex	Somatic index[a]	Standard assay					Assay with ATP				
1	2200	F	6.05	57.60	44.95	7.65	5.77	4.55	53.94	58.71	51.49	13.54	8.99
2	2515	F	7.32	54.49	51.72	11.54	5.32	3.55	57.26	59.71	20.86	7.65	6.77
3	2625	F	7.54	56.33	41.50	19.75	5.99	1.66	54.60	42.17	54.82	41.71	10.43
4	2250	F	7.64	57.26	34.29	12.76	9.10	9.65	63.92	55.04	62.26	55.82	50.61
5	2485	M	1.81	57.93	42.95	5.10	3.99	3.99	54.05	58.49	39.28	13.98	9.76
6	3610	M	2.22	53.82	44.17	12.09	7.54	7.10	56.93	61.10	58.82	58.49	23.41
7	2425	M	4.41	60.48	36.95	12.43	9.21	11.54	65.59	57.82	63.81	55.16	43.72
8	2440	M	5.61	54.27	40.51	6.32	7.32	4.10	67.14	53.05	58.93	36.07	28.74
Mean 1–8				56.32	42.13	10.96	6.78	5.77	59.18	55.84	51.28	35.30	22.80
S.D.				2.26	5.29	4.65	1.84	3.37	5.48	6.14	14.52	21.00	16.98

[a] Weight of gonads expressed as percent of total body weight

Table 3–Statistical comparison of data shown in Table 2

Treatment compared	Probability level (P)[a]	
	Standard assay	Assay with ATP
0 days – 3 days	< 0.001	NS[b]
0 days – 7 days	< 0.001	NS
0 days – 10 days	< 0.001	0.02
0 days – 14 days	< 0.001	< 0.02
3 days – 7 days	< 0.001	NS
3 days – 10 days	< 0.001	NS
3 days – 14 days	< 0.001	< 0.02
7 days – 10 days	NS	NS
7 days – 14 days	NS	< 0.02

[a] Student's t-test
[b] No significant difference (P > 0.05)

has been extensively studied. Saito et al. (1959) found that in rainbow trout stored at 0° the ATP content of the white dorsal muscle decreased from 3.53 μmoles/g to 0.48 after 6 hr and to 0.17 after 24 hr. A similar picture was observed by others with rainbow trout stored without freezing (Tomlinson et al., 1961; Kuusi and Aalto, 1968). Partmann (1972) found that NAD^+, occurring in the white muscle of trout was largely lost after 3 days of storage at +3°C. Jones and Murray (1966) working with chilled cod, *Gadus callarias*, noted that within a few hours of death there was a rapid rise in combined concentrations of NAD^+ and NADH, which was followed by a steady fall during 4 days of storage.

Correlation between lactic acid formation in muscle extracts and duration of storage of sockeye salmon in ice

These experiments were intended to investigate whether the test for lactic acid formation from FDP could be used to distinguish between groups of sockeye salmon stored in ice for different periods of time. In this connection, it was important to know the degree of variation in the rate of deterioration of individual fish. In Table 2 the changes taking place during 2 wk of ice storage are shown separately for eight fish. The determinations were made using both standard and ATP fortified assays. When the standard assay was used the level of activity in the muscle of freshly killed salmon showed little variation between individual fish and there was no noticeable difference due to sex or degree of maturity. During the first week of storage the activity decreased considerably in all the fish tested, amounting to approximately 20% of that found in the freshly killed fish. In the presence of added ATP, the activity remained in most of the salmon unchanged during the first week. After 10 days it showed to decrease in over half of the fish and after 14 days it dropped in all fish with the exception of one. A statistical analysis of the foregoing data (Table 3) indicates that the standard assay enables us to distinguish between groups of freshly killed salmon and that stored in ice for 3–14 days, as well as between a 3-day iced salmon and that held in ice for 7–14 days. On the other hand, by using the assay with ATP it was possible to make a distinction between groups of salmon held in ice for 1 and 2 wk. Although the assay with ATP extends the usefulness of the text beyond the first week of storage, the variations between fish are more pronounced than that with the standard assay. Differences in the rate of deterioration of individuals of the same species were also observed with the other methods for testing the deterioration of the quality of fish (Gould and Peters, 1971). The problem arising from fish-to-fish variation may generally be overcome by sampling a larger number of fish.

In conclusion it appears that the test for lactic acid formation from FDP has a potential value for assessing the degree of freshness of salmonids held for up to 14 days in ice.

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EFFECT OF MOISTURE, CARBOHYDRATE AND ATMOSPHERE ON THE FUNCTIONAL STABILITY OF FISH PROTEIN ISOLATES

INTRODUCTION

DURING THE LAST several years, there has been an increasing interest in using proteins of marine origin for the preparation of concentrates and isolates that possess functional properties (Cobb and Hyder, 1972; Hale, 1972; Meinke et al., 1972; Bligh et al., 1973). Although these and other investigators have described various procedures for preparing functional proteins, there is very little data on the functional stability of these products. Spinelli et al. (1972) reported that they could prepare fish protein isolates by recovering protein-phosphate complexes from slurries of partially hydrolyzed fish proteins. They found that freshly prepared isolates had functional properties, but that these properties gradually deteriorated during storage. They also found that if the isolates were co-dried in the presence of certain carbohydrates, such as corn syrup solids, or if they were stored at refrigerated temperatures, the storage stability of the isolates was enhanced (Spinelli et al., 1973). It has been well documented that chemical and physical reactions responsible for food alteration during storage are governed by such factors as temperature, atmosphere, moisture (water activity) and the reactive nature of the components comprising the food (Acker, 1969; Rockland, 1969; Labuza et al., 1972). The objectives of this study were to determine the influence of these factors on the functional stability during storage of protein isolates prepared from enzymically modified myofibrillar fish proteins (EMMP).

MATERIALS & METHODS

Preparation of protein isolates

Isolates were prepared from partially hydrolyzed myofibrillar rockfish protein as described by Spinelli et al. (1972). When carbohydrates were co-dried with the isolates, aqueous solutions were prepared and mixed thoroughly with the neutralized isolates prior to drying. All isolates were freeze-dried in a Thermovac Model FDC IND 32F freeze dryer. Shelf temperatures were 25°C ± 5°C. Typical samples contained 5–7% moisture and less than 0.1% lipid.

Humidification of the isolates

Saturated salt solutions were used to produce constant relative humidities (Rockland, 1960).

For storage studies, 2–3g samples were placed in polystyrene petri dishes (15 × 90 mm) and equilibrated in vacuum desiccators or glass desiccator jars. The desiccators were stored in a constant-temperature cabinet maintained at 20°C.

Samples for absorption and desorption isotherms were prepared by first equilibrating the isolates for 12 days at RH 0 and RH 88 in vacuum desiccators as described by Labuza et al. (1966). 2-g samples were then re-equilibrated at various relative humidities.

The equilibrium moisture content of the samples was determined by a vacuum oven method (Rockland, 1957). Duplicate samples were dried in a vacuum oven (29 in. Hg, 70°C) to a constant weight.

Storage in air, nitrogen and vacuum atmospheres

After equilibration (at ERH 12), approximately 5g of sample was sealed in a C-enameled (106 × 301) can. For vacuum packing, the can containing the sample was placed in the chamber of a Rooney can sealer and a vacuum of 25 in. of Hg was drawn on the chamber for 2 min prior to sealing. Nitrogen atmospheres were produced by twice releasing the vacuum with N_2 prior to sealing.

Emulsifying capacity

Emulsifying capacity was determined by an electrical resistance method similar to that of Webb et al. (1970). A 1-liter jacketed Waring Blendor cup equipped with electrodes mounted at the base of the cup was used. Blendor speed was 16,000 rpm. Oil was added at a rate of 1 ml per second. All analyses were done in duplicate using 0.5g of sample and 100 ml of water for each determination. Corrections were made for the moisture and carbohydrate content of the samples and the results are given in terms of g oil emulsified per 0.5g protein.

Emulsifying capacity values in excess of 70g

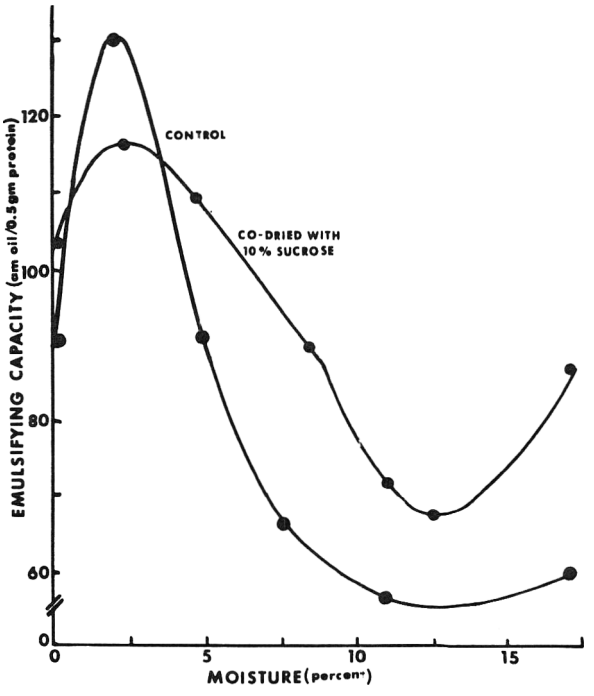

Fig. 1–Effect of moisture and sucrose on the emulsifying capacity (EC) of EMMP stored for 2 months at ambient conditions. Initial EC values of the control sample and the sample co-dried with sucrose were 133 and 118, respectively.

oil/0.5g sample can be reproduced to within ± 2.0g oil/0.5g sample. Emulsifying capacity values below 60g oil/0.5g sample are not considered to be reliable and are obtained only with samples that form poor emulsions.

RESULTS & DISCUSSION

Effect of carbohydrate and moisture

To determine if improved functional stability was dependent on the type of carbohydrate added, EMMP samples were co-dried with carbohydrates (10% dry wt protein) from several different classes. These included mono- and di-saccharides, polyhydric alcohols and corn syrup solids (40 DE). Emulsifying capacities were determined immediately after drying and periodically during storage at ambient temperature and RH.

The percent reductions in EC values after 2 months of storage are presented in Table 1. The least reduction in EC values occurred in EMMP samples co-dried with the disaccharides (12–23%). Reductions in EC values ranging from 30–50% in samples co-dried with polyhydric alcohols, mono-saccharides and corn syrup solids showed that these substances offered little or no protection to the isolates.

Table 1—Effect of various carbohydrates on the emulsifying capacity of EMMP[a]

Carbohydrate source	Emulsifying capacity % reduction	Carbohydrate source	Emulsifying capacity % reduction	Carbohydrate source	Emulsifying capacity % reduction
Control	45	Glucose	35	Sucrose	12
Glycerine	35	Xylose	40	Lactose	23
Mannitol	42	Arabinose	50	Melibiose	19
Sorbitol	32	Corn syrup solids	30	Cellibiose	15

[a] All samples were co-dried with 10% carbohydrates and stored at ambient conditions for 2 months.

The differences in the comparative effects of the sugars may be due in part to the generally more reactive nature of the monosaccharides and protein (Maillard reactions). A distinct color change (from white to yellow) was observed in samples co-dried with monosaccharides and corn syrup solids, but not with samples co-dried with disaccharides or polyhydric alcohols. As noted by Spark (1969), the degree of reactivity of monosaccharides with amino acids also varies (e.g., xylose is more reactive than glucose) which may account for the variations in the protective effect observed for the monosaccharides. The poor protection offered by the nonreactive polyhydric alcohols was somewhat puzzling in view of the protective effect of the disaccharides. In subsequent experiments, however, we found that sorbitol did offer a significant amount of protection when EMMP co-dried with 10% sorbitol was held at ERH values not exceeding 23 (Fig. 2).

When samples of EMMP were equilibrated and stored in an environment controlled with respect to RH and temperature, we found maximum stability was reached when the moisture content of the product was near 2.5% (Fig. 1). Samples equilibrated and stored at zero RH deteriorated to the same degree and approximately at the same rate as samples equilibrated to a moisture level of 5%. Also shown in Figure 1 is the influence of both moisture and sucrose on the EMMP. Samples of EMMP co-dried with 10% sucrose

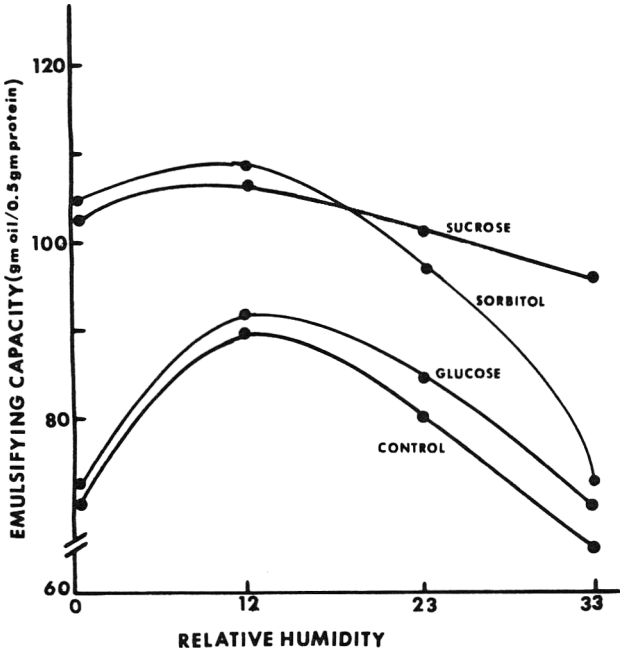

Fig. 2—Emulsifying capacities of EMMP and EMMP co-dried with 10% sucrose, glucose and sorbitol after 6 months of storage at 20°C and at equilibrium relative humidities ranging from 0–33. Initial EC values for the control sample and the samples co-dried with sucrose, sorbitol and glucose were 123, 113, 113,and 115, respectively.

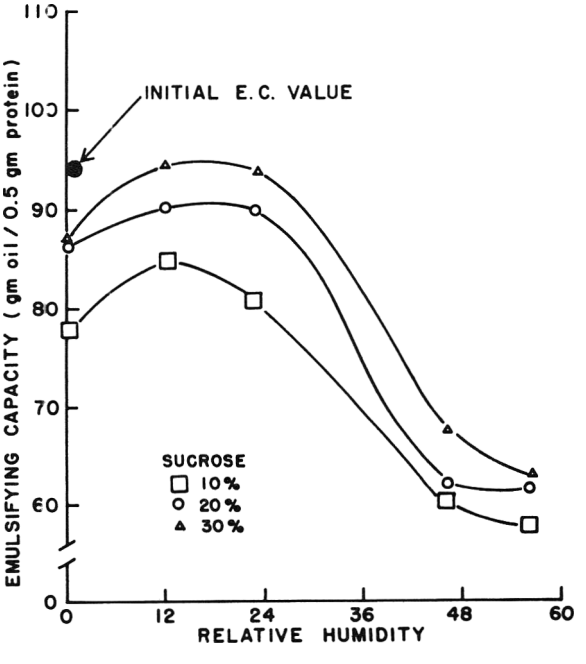

Fig. 3—Effect of sucrose concentrations on the emulsifying capacity of EMMP. All samples were stored for 6 months at 20°C and equilibrium relative humidities ranging from 0–57.

Table 2—Effect of air, nitrogen and vacuum atmospheres on the emulsifying capacity of EMMP and EMMP co-dried with 10% sucrose during storage[a]

		Emulsifying capacity (g oil/0.5g sample)			
		Months in storage			
Sample	Atmosphere	2	3	4	6
Control[b]	Air	91	77	76	65
Control	Nitrogen	93	80	75	66
Control	Vacuum	95	82	80	68
10% sucrose[c]	Air	97	91	91	85
10% sucrose	Nitrogen	96	96	91	84
10% sucrose	Vacuum	101	92	93	90

[a] All samples were equilibrated at RH 12 prior to storage at 20°C.
[b] Initial value 112
[c] Initial value 109

showed a similar pattern of EC loss but the comparative loss was less with respect to the changes in moisture.

As has been shown for other food systems (Salwin, 1959; Rockland, 1957; Martinez and Labuza, 1968), EMMP has an optimum moisture content, deterioration being more rapid both above and below this point.

The instability of the isolates at moisture levels near zero and the observation that the stability is improved by increasing the water content up to a certain level suggests that loss of functionality may be associated with the denaturation of the protein induced by oxidizing lipids (Zerlin and Karel, 1969).

The effect of water and water activity on lipid oxidation has been studied in model systems by several investigators (Tjhio et al., 1969; Heidelbaugh and Karel, 1970; Heidelbaugh et al., 1971; Labuza et al., 1966; Chou et al., 1973; Labuza and Chou, 1974). In general it was found that at very low water activities, lipid oxidation was relatively rapid. The addition of water up to a critical level reduced the rate of lipid oxidation. This was attributed to the hydration of metal catalysts and the hydrogen bonding of peroxide by water. Beyond the critical point, further additions of water resulted in an increased rate of lipid oxidation, which was thought to be due to increased mobility of metal catalysts.

Co-drying proteins with carbohydrates has been found to improve their functional stability and organoleptic quality during storage (Yasui and Hashimoto, 1966; Schultz et al., 1968). This effect has been related to the ability of carbohydrates to inhibit reactions which lead to the denaturation of proteins (Kline et al., 1964). As water activity has been shown to affect the rate of many of these reactions, the protective effect of sucrose could be attributed to its ability to alter the water activity of the system.

Effect of carbohydrates during prolonged storage. EMMP samples co-dried with 10% sucrose, glucose and sorbitol were stored for 6 months at 20°C at equilibrium relative humidities (ERH) of 0, 12, 23 and 33. The sample co-dried with sucrose exhibited the best storage stability over the ERH range (Fig. 2). The decrease in EC after 6 months for this sample was 9, 5, 10 and 15% at ERH 0, 12, 23 and 33, respectively. The loss of EC in the sorbitol sample was slightly less than that of the sucrose sample at ERH 0 and 12 (7 and 3%). As the ERH increased from 12 to 33, the EC of the sorbitol sample decreased to 14% at ERH 23 and 36% at ERH 33. The sample containing glucose showed a loss in EC similar to that of the control. Both the control and the glucose-containing samples exhibited maximum stability at ERH 12, but even at this ERH they lost 27% of their original EC, and at ERH 0 and 33, they lost approximately 45% of their original EC.

Effect of sucrose concentration. The effect of sucrose concentration on the functional stability of EMMP was determined by co-drying the EMMP with 10, 20 and 30% sucrose. These samples were then equilibrated and stored at ERH conditions ranging from 0–57 for 6 months at 20°C. After the end of that time, the sample co-dried with 30% sucrose and stored at ERH 12 and 23 showed no loss of EC. At the same ERH, the sample co-dried with 20% sucrose lost approximately 5% of its EC. The sample co-dried with 10% sucrose showed about 10 and 13% loss of EC at ERH 12 and 23, respectively (Fig. 3).

Effect of atmosphere. No significant differences were found in the rate of loss of EC when EMMP was stored in either an air or nitrogen atmosphere (Table 2). Samples that were stored under vacuum showed slightly less loss of EC than the air- or nitrogen-packed samples. Samples of EMMP co-dried with 10% sucrose and stored under the same conditions of atmosphere and ERH as the control samples showed slightly less but relatively similar losses of EC during storage, indicating that atmosphere plays only a minor role in the functional stability of the protein isolates.

Effect of carbohydrate on moisture absorption and desorption. Figure 4 shows the moisture absorption and desorption isotherms of EMMP and EMMP co-dried in the presence of 10% sucrose, glucose and sorbitol.

Co-drying EMMP with carbohydrate significantly changes the moisture isotherms not only in relation to the control sample but also with relation to one another. We found that when EMMP was co-dried with sucrose the absorption isotherm became more distinctly sigmoidal and there was a pronounced flattening of the isotherm between ERH 12 and 33. The desorption isotherm showed a similar shape. As is common in such phenomena, desorption-absorption curves showed marked hysteresis. With glucose, there was no significant change in moisture absorption between ERH 23 and 33; the desorption isotherm showed that there was no moisture loss between RH 23 and 12. The sorbitol isotherm had a linear configuration from ERH 0–33, and then showed no significant change until the ERH exceeded 44. The desorption isotherm was typically sigmoidal with the flattest portion of the curve between ERH 33 and 23.

The relation between the stability (EC) of the samples and ERH is not easy to discern for all the samples. It must be assumed that the instability of all samples at ERH 0 is due to an oxidation occurring in the product. As early as 1956, Tappel had shown that proteins oxidized and that this oxidation was an important deteriorative mechanism for the loss of functional and biological properties in freeze-dried proteinaceous materials. Even at the low lipid contents present in these samples, the possibility of lipid-induced peroxidation of the protein could proceed as postulated by Zerlin and Karel (1969). At higher ERH's, water can act as a medium for the propagation of chemical and physical reactions (Rockland, 1969; Labuza et al., 1971). The samples containing glucose and sorbitol, however, show maximum stability at ERH 12, a point at which the isotherm is changing very rapidly with respect to moisture. It would appear therefore that either the water in these samples is preferentially bound to the carbohydrate instead of the reactive sites of the protein or that the increased hydration causes a different rate of swelling (Chou et al.,

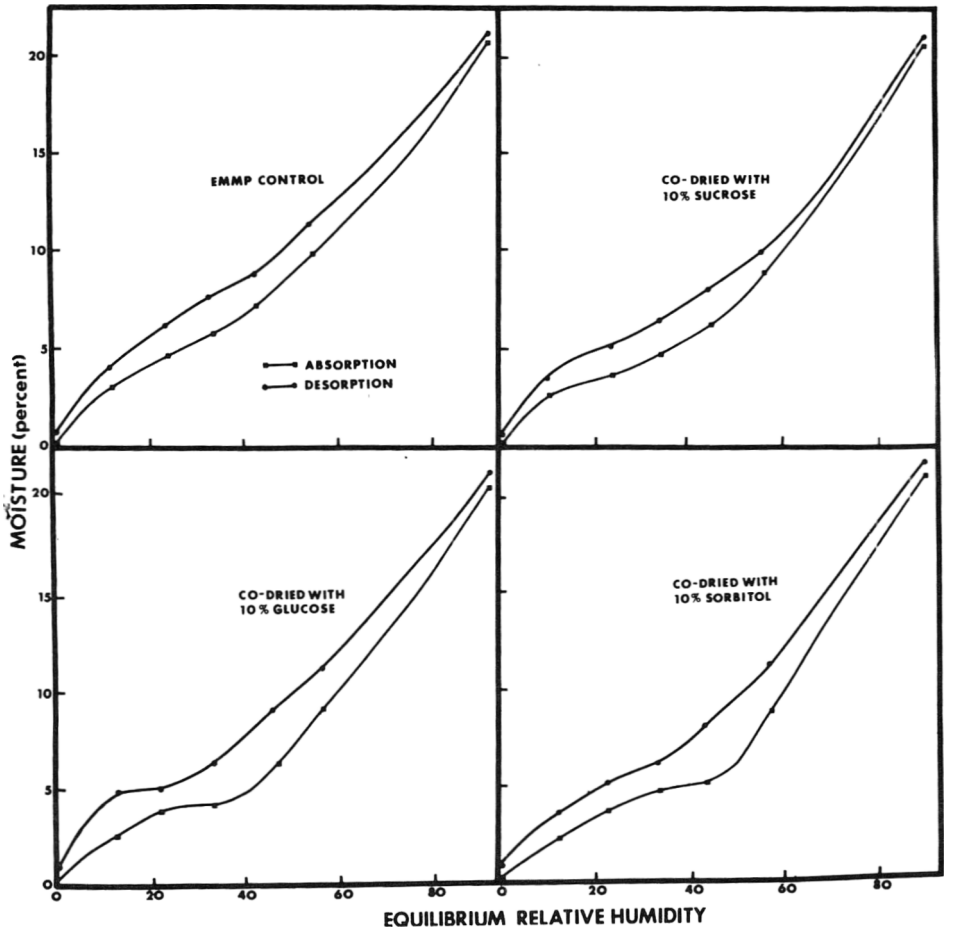

Fig. 4—Absorption and desorption moisture equilibrium curves for EMMP and EMMP co-dried with 10% sucrose, glucose and sorbitol.

1973) that could change the mobility of catalytic constituents in the system.

SUMMARY & CONCLUSIONS

THE ABOVE STUDIES showed that the functional stability (as reflected by changes in emulsifying capacity) of fish protein isolates is closely related to the moisture content and water activity of the isolates. Fish protein isolates exhibited maximum stability at moisture contents near 2.5%. At moisture contents above or below 2.5%, there was a rapid decline in the EC of the isolate during storage. The water activity of the isolates could be lowered by co-drying the isolates with various types of carbohydrates. Only nonreducing sugars, however, significantly broadened the range of moisture contents at which the stability of the isolates was enhanced during storage. Isolates co-dried with 10% sucrose having a moisture content of 2.5–4.0% lost from 15 to 20% of their emulsifying capacity during 6 months of storage at 20°C. Under the same storage conditions, isolates co-dried with 30% sucrose lost none of their emulsifying capacity during the 6-month storage period. The storage stability of the isolates could be enhanced by co-drying with 10% sorbitol if the moisture content did not exceed 2.5%. Isolates co-dried with glucose displayed evidence of non-enzymic browning during storage and rapidly lost their EC regardless of their moisture contents (0–10%).

Isolates stored under air, vacuum and nitrogen atmospheres and equilibrated to identical moisture contents lost their EC at comparatively similar rates during storage. These experiments showed that atmosphere plays a minor role in its effect on the functional stability of the isolate.

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Use of trade names in this article does not imply endorsement.

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PREPARATION AND EVALUATION OF SOY-FORTIFIED GLUTEN-FREE BREAD

INTRODUCTION

FOR INDIVIDUALS suffering from gluten-intolerance e.g., celiac-sprue, exclusion of wheat bread and other gluten-rich products from the diet becomes necessary (Kasarda, 1972; Van Dekamer and Weijers, 1965). Gluten-intolerance is perhaps related to the absorption of a toxic peptide which results from the gliadin fraction of gluten which normally is hydrolyzed thus causing malabsorption, diarrhea and other gastro-intestinal disturbances (Kasarda, 1972; Van Dekamer and Weijers, 1965; Dissanayake et al., 1973). Cereals lacking-or-low in gluten e.g., corn, rice etc. have usually been considered nontoxic to celiac patients (Kasarda, 1972). As a result, breads have been made with nonwheat flours and starches as a substitute for wheat flour. Gluten-free flours, however, fail to form a continuous phase and thus lack the necessary dough structure for the production of a good quality bread. Breads produced are often course and crumbly, poor in volume and over-all quality and thus are poorly accepted (Bowman et al., 1973; Goertz et al., 1965; McGreer, 1967). Some improvement in bread quality is obtained when gums and surface-active agents are used to form a stable dough structure (McGreer, 1967; Steele et al., 1965; Kim and DeRuiter, 1968); xanthan gum has been found to be most effective in this regard (Kulp et al., 1974; Christianson et al., 1974). Present work reports on a gluten-free bread of markedly improved quality that also contains substantial amounts of protein.

EXPERIMENTAL

SOY PROTEIN ISOLATE containing 88% protein (N × 6.25), 0.1% fat, 4.3% ash, and 4.9% moisture and unmodified wheat starch were used to make experimental breads. Patent flour from hard red winter wheat was used for the control. Fortification of starch with soy was carried out at three levels—20, 30 and 40%. Table 1 lists the formulae used. White bread was made by a straight-dough procedure by mixing all ingredients for 1 min at no. 1 speed (rpm, 60), 1 min at no. 2 speed (rpm, 106) and for 13 min at no. 4 speed (rpm, 311) in a Hobart M-800 mixer using bowl (40 qt) and hook. Following a fermentation period of 90 min, dough was punched and fermented for an additional 30 min, scaled at 18½ oz, intermediate proofed for 10 min, molded, proofed to template height (59 min), and baked (218°C, 25 min). Starch bread was made by adding yeast (suspended in 43°C water) and melted shortening to dry ingredients (premixed for few seconds) and mixing for 1 min at no. 1 speed (rpm, 139), and for 5 min at no. 2 speed (rpm, 285) in a Hobart N-50 3-speed mixer using bowl and paddle. The resultant batter was fermented for 30 min at room temperature (about 21°C), remixed for 1 min at no. 1 speed and for 4 min at no. 2 speed. 16 oz of batter was then poured into an aluminum foil loaf pan (2½ × 4 × 8 in), gently shaken to evenly distribute it, proofed (32, 27, 31 and 30 min at 0, 20, 30 and 40% soy levels, respectively) and baked (218°C, 20 min).

A series of bake tests were conducted to determine the water requirement of the dough (wheat bread) and of the batter (starch bread); values thus determined are shown in Table 1. Following baking and allowing a cooling period of 1 hr, all loaves were plastic-bagged. Next morning they were removed from the bags, weighed and their volume (rapeseed displacement method) determined. Loaves were then sliced and their quality assessed by the baking technologist (Table 2).

A separate set of loaves was baked for nutrient analyses and for the determination of protein quality by rat-feeding studies. Protein, moisture, and fat contents were determined on air-dried, finely ground samples by the standard AOAC methods (1970). Protein quality was assessed based on protein efficiency ratios (PER) determined by the AOAC method (1970). Individually housed, weanling male rats (Sprague-Dawley) were fed test diets for 4 wk. The weekly gain in weight and diet intakes were recorded. A casein diet served as the reference control. PER was calculated from increase in weight of rats in 4 wk (g)/total protein (g) consumed; determined and corrected (casein, 2.5) values are reported in Table 3.

RESULTS & DISCUSSION

NONWHEAT FLOURS or starches usually result in poor quality bread because of the lack of structure-forming proteins (Kasarda, 1972; Goertz et al., 1965). Gel-forming substances and surface-active agents usually stabilize the dough structure and improve bread quality (McGreer, 1967; Kim and DeRuiter, 1968; Jongh, 1961); this effect is most noticeable in the presence of nongluten proteins e.g., egg, milk etc. This, however, increases the cost of production substantially. To eliminate this and also to obtain a high-protein product, a number of soy protein preparations were tested for their dough-stabilizing properties. Preliminary studies showed that soy flours and soy protein concentrates could not be used at high levels without severely decreasing bread quality. Soy protein isolate, on the other hand, performed quite satisfactorily and was used in these studies. Apparently it functions differently than soy flours and concentrates. Results in Table 2 show

Table 1—Bread formula

Ingredients	Wheat bread (parts per 100 parts flour or starch)	Starch bread (parts per 100 parts flour or starch)
Wheat flour or wheat starch[a]	100	100
Soy protein isolate[b]	0	0, 20, 30, 40[d]
Xanthan gum[c]	0	2
Sucrose	5	14
Sodium chloride	1.5	2
Vegetable shortening	3	10
Yeast, compressed	3	7.5
Yeast food	0.5	0
Monoglycerides	0.5	0
Water	63	120, 155, 180, 195[d]

[a] Wheat starch (Aytex P) from General Mills, Inc.
[b] Soy protein isolate (Promine D) from Central Soya Co., Chicago
[c] Xanthan gum (Keltrol) from Kelco Co., Chicago
[d] Values corresponding in sequence shown. Warm (43°C) water used for starch breads

Table 2—Bread characteristics and composition

	Starch bread				Wheat bread
Soy (%)	0	20	30	40	0
Characteristics[a]					
Loaf weight (oz)	14.50	14.25	14.33	14.60	16.25
Loaf volume (ml)	1587	1875	1675	1633	2908
Specific loaf volume (cu in/oz)	6.68	8.03	7.13	6.82	10.92
Break and shred[b]	good^{-}	good^{++}	good^{+}	good	good^{+}
Appearance	good^{+}	good	good^{++}	good^{+}	good^{+}
Crust color	light	slightly dark	dark	dark	light
Crumb color	bright	bright	slightly dull	slightly dull	bright
Grain	poor	good^{++}	good^{+}	good	good^{+}
Texture	poor	excellent	good^{++}	good	good^{+}
Flavor	poor	good	good^{-}	good^{-}	good^{+}
Mastication	rubbery, crumbly	good, slightly crumbly	good^{+}	good^{+}	good^{+}
Composition (separate bake)[c]					
Fresh loaf weight (g)	406.3	403.5	405.5	407.8	450.0
Moisture (%)	48.4	50.8	52.8	53.2	35.8
Protein (%)[d]	0.69	7.06	9.17	11.10	8.24
Protein (%)[e]	0.86	9.21	12.47	15.22	8.24
Ether extract (%)	3.99	2.36	2.22	2.01	1.76

[a] Average of four loaves
[b] No shred in starch breads
[c] Expressed on fresh-loaf basis unless indicated otherwise
[d] N X 6.25 except N X 5.7 for wheat
[e] On equivalent-moisture basis

that loaf volume and internal characteristics of starch bread were markedly improved by the addition of 20% soy protein isolate, and although higher levels of soy decreased bread quality somewhat, bread of desirable characteristics was obtained at even 40% level. Water-binding, gelling and stabilizing properties of soy appeared to be resistant to disruptions at baking temperatures (Fig. 1; Table 2). With the addition of soy, crust and crumb color of starch breads tended to be slightly darker but not objectionable. Grain and texture showed a marked improvement from a rough, crumbly, open-faced interior to a more tender, close-grain even texture, with a compact cell-structure comparable to that of the control bread (Fig. 1). Crumbliness greatly decreased and then disappeared as the level of soy added increased. No off-flavor was detected and all soy-based breads were normal (bland) to taste which increased their over-all acceptability. Preliminary studies showed that the use of shortening, yeast, gum and soy in amounts exceeding that used (Table 1) did not improve bread quality further and that strict adherence to water requirements of the batter is imperative to the production of good quality bread. Sucrose at the level used added desirable flavor but not the sweetness.

Compared to the control bread, all starch-breads were significantly higher ($P < 0.01$) in water content which increased

Table 3—Protein efficiency ratio—PER

	Diet				
	Casein	Wheat bread	Starch bread		
Soy (%)	0	0	20	30	40
Food intake (g)	301 ± 23	207 ± 24	201 ± 17	192 ± 15	208 ± 18
Weight gain (g)	86.1 ± 9.4	16.5 ± 3.3	23.0 ± 2.8	23.3 ± 2.8	26.0 ± 3.6
PER (measured)	3.17 ± 0.15	0.89 ± 0.18	1.27 ± 0.09	1.35 ± 0.10	1.39 ± 0.09
PER (corrected)	2.50 ± 0.12	0.70 ± 0.14	1.00 ± 0.07	1.06 ± 0.08	1.09 ± 0.06

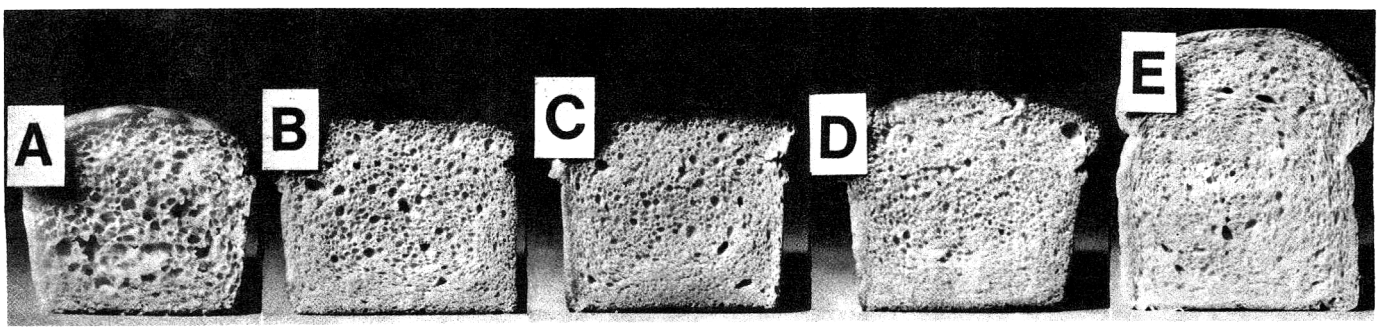

Fig. 1—Internal profile of breads. A, starch bread (soy, 0%); B, starch bread (soy, 40%); C, starch bread (soy, 30%); D, starch bread (soy, 20%); and E, control wheat bread.

somewhat with the level of soy used probably because more water was initially required to bake an acceptable bread (Tables 1 and 2). On equivalent-moisture basis, all soy breads were higher in protein content than the wheat bread; fat content was also significantly higher ($P < 0.01$) in starch-breads. Fat recovery (ether extraction) decreased when soy was added to starch bread because soy probably exerted a fat-binding effect during breadmaking. Protein quality (PER) of soy-fortified starch breads was significantly higher ($P < 0.01$) compared to wheat bread (Table 3). This occurred in spite of the fact that PER of soy isolate is invariably low and quite variable as compared to soy flours and concentrates. This is due to variations in the techniques used in the preparation of soy isolate and consequently variable losses that occur of the most limiting amino acids (methionine and cystine) and of lysine (Kim and DeRuiter, 1968; FAO, 1970; Central Soya Co., 1973; Sipos et al., 1974).

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EVALUATION OF PROTEIN QUALITY OF IRRADIATED FOODS USING Tetrahymena pyriformis W. AND RAT ASSAY

INTRODUCTION

WHOLESOMENESS of radiation processed foods aimed at assessing freedom from toxicity is usually inferred from multi-generation feeding trials with experimental animals similar to those for carcinogens and food additives (WHO, 1966; 1970). Though protein quality, a major determinant of nutritive value in many foods, is conventionally measured using animals, microbiological assay techniques using organisms having high proteolytic activity like *Streptococcus faecalis* (Halvey and Grossowicz, 1953), *Streptococcus zymogens* (Ford, 1962) and *Clostridium welchii* (Boyd et al., 1948) have been developed. *Tetrahymena pyriformis,* a ciliated protozoan, also possess an active proteolytic enzyme system (Dickie and Leiner, 1962; Boyne et al., 1967) and can utilize intact native protein for growth (Rockland and Dunn, 1946). Its amino acid requirement is similar to that for growing rats (Kidder and Dewey, 1961). In fact, results obtained on growth response to a variety of protein sources in the assay medium have been shown to be comparable to conventional biological value and protein efficiency determinations with the rat (Rosen and Fernell, 1956; Stott et al., 1963).

The present work relates to a simple approach, as an alternative to the rat, for determination of protein efficiency in foods with attendant advantages of convenience, rapidity in testing and inexpensiveness. *Tetrahymena pyriformis* was the organism of choice because of its cell size, permitting ease in counting. Changes in protein quality, if any, in irradiated foods, with special reference to shrimp and wheat, were assessed using *Tetrahymena.* Additionally, the growth responses of the organism, grown on irradiated foods as protein source for several transfers, were determined. The effects of radiation on protein quality was simultaneously evaluated using the rat assay procedure (Allison, 1964).

MATERIALS & METHODS

FREEZE-DRIED, dehydrated, canned and semi-dried shrimp samples, gamma-irradiated in-package irradiator in the range 0.25–0.32 Mrad (dose rate 0.25 Mrad/hr; overdose ratio 30%) in air, vacuum or nitrogen atmosphere, were prepared according to the methods described by Srinivas et al. (1974). Procedures for isolation of individual protein components from wheat flour, namely, gluten, gliadin and glutenin and for irradiation, etc. were the same as described earlier (Srinivas et al., 1972). Samples were irradiated at ambient temperature (28–30°C with three doses in the dose range 0.020–0.026, 0.20–0.26 and 1.0–1.3 Mrad (overdose ratio about 30%)). The irradiated products were used in the experiments after 1–2 wk of storage at 0–4°C.

Maintenance of stock culture of Tetrahymena

Stock culture of *T. pyriformis,* wild strain, was obtained through the courtesy of Dr J.E. Ford of the National Institute for Research in Dairying, Shinfield, Reading, U.K. This was maintained on 2% proteose-peptone (Difco) sterile media (Stott et al., 1963) and grown in the dark at 25°C. The organism was subcultured regularly at weekly intervals.

Assay procedure for Tetrahymena

Suspensions of ether-extracted test material (60 mesh) were made into appropriate media (10 ml, pH 7.8) with other nutrients required for maximal growth. Composition of the medium was essentially the same as described by Rosen and Fernell (1956) and modified by Stott et al. (1963) except for individual nucleotides, which were replaced by equivalent amount of yeast RNA. This was hydrolyzed with 0.3N NaOH at 37°C for 18 hr, passed through Dowex-50 H^+ column (0.9 × 5 cm) and added to the medium.

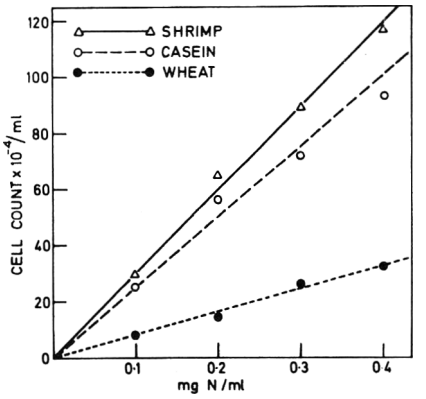

Fig. 1—Growth response of T. pyriformis to different protein sources: Suspensions of test proteins were made into media at graded levels (0.1–0.4 mg N/ml). Cells were counted after 4 days of incubation.

Experimental culture containing test protein (0.1–0.4 mg N/ml) was inoculated aseptically with 5–6 × 10^4 cells in log phase from broth culture and incubated under air at 25°C for 4 days in the dark with frequent shaking. Growth was arrested by killing the cells with 10% formalin. Cells were counted using Neubauer ruled double cell haemocytometer. In each experiment, the final population/ml of the medium was calculated by multiplying the mean values of eight individual counts in 1 mm^2 area, with a factor of 2 × 10^4. All cell counts reported represent averages of two experiments carried out in quadruplicate. The standard error of the mean values were throughout less than 10%.

Composition of rat diets

Wheat, unirradiated or irradiated at 20 or 200 Krad, was included at the 75% level in diets, supplemented with necessary components to ensure nutritional adequacy (Table 1A). The 200 Krad treatment provided a nearly tenfold margin over the dose for disinfestation. The diet provided 19.6% protein.

For the 10% wheat protein diet (Table 1B), all the ingredients were thoroughly mixed with finely cut vegetables and water, cooked for 30 min, vacuum dried at 50°C and powdered. Diet was divided into three batches, one serving as control and two others being irradiated at 20 or 200 Krad.

Semi-dried shrimp, unirradiated or irradiated at 0.25 Mrad and stored for 20 and 90 days, respectively (corresponding to two-thirds of their keeping time) (Gore et al., 1970), were used for the animal feeding experiment. The dose employed for radurization ensured nutritional adequacy and microbiological safety of the product. The product was reconstituted for 2–3 hr in water, cooked for 20 min, dried and mixed at 25% level as a protein source (19%) in the diet (Table 1C) with other appropriate additions for adequacy.

For 10% shrimp protein diet, 12.5% shrimp powder in the 25% shrimp diet (Table 1C) was replaced by an equivalent quantity of starch. The casein diet was prepared by substituting 12.5% each of casein and starch in place of 25% shrimp. Dietary proteins were measured by Kjeldahl analysis.

Animal feeding procedures

In each experimental series, Wistar strain albino rats of both sexes, 24 in each group, were fed ad libitum.

Weanling rats, weighing about 35–40g were fed wheat (Table 1A) or shrimp (Table 1C) protein diets. After 1 wk adjustment, food intake and growth of each individual rat, was measured up to 8 wk and food efficiency calculated.

In a separate experiment to assess the protein efficiency of these two irradiated products, young adult rats (100g body weight) were fed diets containing 10% protein. Food consumption and gain in body weight were measured for 4 wk and protein efficiency calculated.

RESULTS

Growth pattern of T. pyriformis W.

The growth pattern of *T. pyriformis* W. resembles that of most other microorganisms. Under the experimental conditions, cells remained in the log phase for 18 hr, followed by exponential growth period (log phase) for another 72 hr, prior to entering the stationary phase. A linear growth response was observed (Fig. 1) when 0.1—0.4 mg N/ml of protein source, casein, shrimp or wheat was added to the medium; the slope of the line was characteristic for each protein. In subsequent experiments, cells were grown in media containing 0.3 mg N/ml for 4 days.

Growth response of T. pyriformis W. to irradiated wheat proteins

Results on changes, if any, in protein quality of irradiated wheat or of isolated proteins therefrom, evaluated using *Tetrahymena*, are given in Table 2. With casein as protein source, the final population was 69 x 10^4 cell counts/ml, whereas with irradiated (0.02—1 Mrad) whole wheat flour or with isolated wheat proteins, the numbers varied between 16 to 28 x 10^4 cell counts/ml and were comparable with their respective unirradiated controls.

The poor growth response of *Tetrahymena* to wheat proteins could be attributed to deficiency in its essential amino acids. It was observed (Table 3) that supplementation of wheat flour with the limiting amino acids, L-lysine, DL-methionine and L-threonine, based on the amino acid pattern for casein singly or in combination, resulted in better growth of

Table 1—Composition of rat diets

A (Wheat diet) (19.6% protein)		B (Wheat diet) (10.0% protein)		C (Shrimp diet) (19% protein)	
Ingredients	%	Ingredients	%	Ingredients	%
Whole wheat flour	75.0	Wheat flour	60.0	Starch	62.0
Casein[a]	13.0	Red gram (*Cajanus cajan*)	4.0	Shrimp powder	25.0
Vitaminized sucrose[b]	5.0	Milk powder	2.0	Vitaminized sucrose[b]	5.0
Sesame oil[c]	5.0	Starch	16.0	Sesame oil[c]	6.0
Sodium chloride	1.5	Sugar	5.0	Sodium chloride	1.5
Calcium lactate	0.5	Non-leafy vegetables (Brinjal and okra)	7.5	Calcium lactate	0.5
	100.0	Leafy vegetables (Spinach and Amaranthus)	1.5		100.0
		Salt	1.5		
		Sesame oil	2.5		
			100.0		

[a] Casein was added in an amount to bring the total protein in the diet to about 20%.
[b] This provided in mg/100g diet, thiamine 0.2, riboflavin 0.4, pyridoxine 0.4, choline 100.0, inositol 100.0, p-amino benzoic acid 30.0, nicotinamide 10.0, calcium pentothenate 1.0, folic acid 0.25, vitamin B_{12} 0.005, biotin 0.01 and menadione 0.5.
[c] 1g of oil was fortified with 5 mg vitamin E, 400 IU, vitamin A and 200 IU vitamin D.

Table 2—Effect of irradiation on protein quality of wheat using T. pyriformis W.[a]

Protein source	Control	Mrad 0.02	0.2	1.0
	Cell count X 10^4/ml			
Casein	69.2	—	—	—
Wheat flour	25.6	24.0	25.0	28.6
Gluten	20.4	21.1	23.8	20.4
Gliadin	16.0	18.0	17.0	17.6
Glutenin	24.0	23.8	24.4	26.2

[a] Whole wheat flour or isolated wheat proteins were gamma-irradiated at 0.02—1.0 Mrad and suspended in medium (0.3 mg N/ml) as protein source. Growth was measured after 4 days. Organisms were killed with 10% formalin and counted under a light microscope. Each value represents the average of two independent experiments, carried out in quadruplicate. The standard error was less than 10%.

Table 3—Effects of supplementation of limiting essential amino acids on wheat protein quality[a]

Protein source	Control	Mrad 0.02	0.2	1.0
	Cell count X 10^4/ml			
Casein	70.6	—	—	—
Wheat flour	28.0	30.0	29.5	27.5
Wheat flour + L-lysine	66.7	67.3	66.0	63.0
Wheat flour + DL-methionine	37.0	38.3	39.3	41.0
Wheat flour + L-threonine	43.5	46.2	47.5	43.2
Wheat flour + L-lysine + DL-methionine + L-threonine	81.0	82.8	78.0	79.0

[a] The organism was grown in medium containing wheat flour, unirradiated or irradiated (0.02—1 Mrad) as protein source. Essential amino acids were supplementec to their levels in casein. Organisms were counted on the 4th day after inoculation. Other details as under Table 2.

the organism. Maximum response was seen with lysine; growth rate exceeded that of casein in the presence of all these amino acids. Organisms grown in media containing irradiated proteins showed no adverse effects.

In similar studies, protein quality in fresh, freeze-dried, semi-dried or dehydro-irradiated shrimp as protein source, was determined and the results are presented in Table 4. Growth with shrimp proteins was better than that with casein. No impairment in the growth of the organism reared on shrimp irradiated in different atmospheres, like air, vacuum or nitrogen, was observed; the final population obtained was almost the same in all the groups. Storage of unirradiated and irradiated shrimp up to 1 and 3 months, respectively, at ambient temperature (25–27°C), had not appreciably affected protein quality.

Transfer studies

In another study, the efficacy of the organism to grow on irradiated foods during several transfers, was assessed. Results (Table 5) on cell population, counted 4 days after each of four successive transfers, did not reveal any adverse effects on growth of the organism.

Assessment of food and protein efficiency using rat

Comparative growth data at the end of 8 wk on rats fed unirradiated or irradiated wheat or shrimp diets (Table 6) showed no significant differences in body weight gain or food efficiency ratio between the respective control and experimental groups. There was no appreciable change in protein efficiency (Table 7)

Table 4—Evaluation of protein quality of processed and stored shrimp using Tetrahymena pyriformis W.[a]

Protein source	Storage time in days			
	0	30	60	90
	Cell count X 10^4/ml			
Casein	72.2	–	–	–
Freeze-dried shrimp	92.1	89.5	90.5	89.2
Dehydrated shrimp (2.5–3.0% moisture)	84.0	85.1	82.3	83.4
Canned shrimp	88.3	89.8	87.1	85.5
Dehydro-shrimp (40% moisture)	89.5	91.7	–	–
Dehydro-shrimp, irradiated (0.25 Mrad) in air	91.8	88.5	89.7	92.5
in vacuum	94.3	90.1	89.0	88.6
in nitrogen	92.4	90.0	88.5	89.2

[a] Shrimp samples, either freeze dried, dehydrated or processed as described earlier, were stored at ambient temperature (25–27°C) for 1–3 months. At indicated time intervals, samples were removed, ground and added as protein source to media. Other details as under Table 2.

Table 5—Growth of Tetrahymena pyriformis W. on irradiated foods (Transfer studies)[a]

Protein source	Transfer			
	I	II	III	IV
	Cell count X 10^4/ml			
Casein	66.5	65.0	65.6	68.2
Wheat flour, unirradiated	27.5	25.5	28.2	29.5
Wheat flour, irradiated 20 Krad	25.0	28.0	27.2	26.0
Wheat flour, irradiated 200 Krad	26.0	27.7	26.8	24.0
Wheat flour, irradiated 1 Mrad	26.2	27.6	25.1	28.0
Dehydro-shrimps	92.0	88.5	89.6	93.4
Dehydro-irradiated shrimps (0.25 Mrad)	90.5	92.3	87.5	89.6

[a] Irradiated materials were stored for 1–2 wk at 0–4°C. Cells were grown for 4 days and transferred to fresh medium four times. Cell population was counted after 4 days of each transfer. Other details as under Table 2.

Table 6—Food efficiency ratio of rats fed unirradiated and irradiated wheat or shrimp diets

Diet	Sex	Initial body wt (g)	Body wt. after 8 wk (g)	Increase in body wt. (g)	Total food intake for 8 wk (g)	Food efficiency ratio[a]
Wheat irradiated						
Control	Male	51.1 ± 1.8	210.7 ± 8.8	159.6 ± 8.6**	623.0	0.25
0 Krad	Female	46.1 ± 2.2	199.4 ± 5.8	153.3 ± 5.6***	584.9	0.26
20 Krad	Male	49.1 ± 1.4	215.0 ± 1.8	165.9 ± 2.2**	630.5	0.26
	Female	43.1 ± 2.8	204.1 ± 3.4	161.0 ± 3.6*	600.0	0.27
200 Krad	Male	50.3 ± 4.2	215.0 ± 4.8	164.7 ± 6.5**	616.0	0.26
	Female	44.1 ± 2.3	196.5 ± 4.6	152.4 ± 3.1*	582.6	0.26
Shrimp						
Control	Male	44.5 ± 2.6	290.4 ± 8.9	245.9 ± 7.0*	696.1	0.35
0 Krad	Female	43.2 ± 1.2	246.0 ± 4.0	202.8 ± 4.5*	621.5	0.32
0.25 Mrad	Male	50.0 ± 0.9	284.8 ± 2.1	234.8 ± 3.3*	717.0	0.33
	Female	48.4 ± 1.5	236.4 ± 6.3	188.0 ± 7.1	646.0	0.29

[a] Food efficiency ratio was calculated as g increase in body wt/g food intake. Each value is the average of 12 determinations ± SE.
* $P < 0.3$; ** $P < 0.7$; *** $P < 0.9$.

Table 7—Protein efficiency of irradiated foods by rat assay

Diet	Sex	Initial body wt (g)	Body wt after 4 wk (g)	Increase in body wt (g)	Food intake for 4 wk (g)	Protein intake	Protein efficiency[a]
Casein (Comparison diet)	Male	100.3 ± 1.2	176.2 ± 1.0	75.9 ± 1.3	331.0	33.1	2.3
	Female	100.8 ± 0.8	174.5 ± 0.4	73.7 ± 0.8	308.0	30.8	2.4
Wheat (unirradiated)	Male	100.8 ± 0.7	143.8 ± 2.0	43.0 ± 0.9**	275.2	27.5	1.5
	Female	100.3 ± 1.1	142.5 ± 1.5	42.3 ± 1.2**	291.8	29.2	1.4
Wheat (irradiated at 20 Krad)	Male	100.5 ± 1.0	142.7 ± 1.7	42.2 ± 1.8***	284.0	28.4	1.4
	Female	100.0 ± 0.6	140.3 ± 1.3	40.3 ± 1.0*	268.5	26.8	1.5
Wheat (irradiated at 200 Krad)	Male	100.7 ± 1.5	142.8 ± 1.2	42.1 ± 0.9**	275.7	27.6	1.5
	Female	100.3 ± 0.9	143.9 ± 1.6	43.6 ± 0.7**	292.9	29.3	1.5
Shrimp (unirradiated)	Male	100.3 ± 0.6	192.1 ± 0.4	90.7 ± 1.1**	281.4	28.1	3.2
	Female	100.6 ± 0.9	195.1 ± 0.6	94.7 ± 3.6*	272.5	27.3	3.4
Shrimp (irradiated at 0.25 Mrad)	Male	100.4 ± 1.0	195.6 ± 1.0	93.5 ± 1.6**	276.2	27.6	3.3
	Female	100.3 ± 1.1	188.6 ± 0.9	87.3 ± 4.0*	273.2	27.3	3.2

[a] Protein efficiency was calculated as gain in body weight/protein consumed. Results are averages for 12 male and 12 female rats in each group ± SE.
* $P < 0.3$; ** $P < 0.5$; *** $P < 0.7$

between the groups; however, it was higher for shrimp compared to reference protein casein. The values were low with wheat, because of its inferior amino acid composition.

DISCUSSION

MULTI-GENERATION FEEDING trials with animals, employing the elaborate and established protocols to evaluate the potential toxicity, if any, of irradiated foods (WHO, 1966) have demonstrated that irradiated wheat (Aravindakshan et al., 1970) and dehydro-irradiated shrimp (Vakil et al., 1973) are wholesome. Though microorganisms have been used to study the effects of radiation, protozoa in general and *Tetrahymena* in particular, may be a better choice because of more nearness in physiological processes to those of mammals. Lower cell counts with wheat proteins compared to casein (Table 2) suggest that growth response of the organism correlates well with protein quality in media. Lowest counts (16–18 x 10^4 cells/ml) with gliadin is in conformity with its inferior quality. The slightly lower counts with gluten compared to whole wheat flour may be due to its low available lysine value (Srinivas *et al.*, 1972). Bunyan and Woodham (1964) have established a distinct correlation between available lysine content and nutritive value of fish meal using *T. pyriformis*. The improved growth on addition of the limiting amino acids to wheat flour (Table 3) suggests that the organism is sensitive to nutritional stress and has specific requirements of essential amino acids for optimum growth.

Growth with shrimp protein was found better than that with casein (Table 4); results of rat experiments gave PER value of 3.3 for shrimp protein as compared to 2.3 for casein (Table 7). Similar good correlation between the results for protein quality using *Tetrahymena* and biological assays (Kamath and Ambegaokar, 1968) has been reported. No differences in the final population of the protozoan were observed when irradiated wheat or shrimp was added to the medium as compared to the corresponding unirradiated samples. This is borne out in the studies with rats where it is observed that food efficiency ratio (Table 6) and protein efficiency (Table 7) of these products are not affected by irradiation. Reduced growth of *Tetrahymena* on irradiated (1–2.3 Mrad) synthetic media was attributed not to any toxic factors but to the losses of vitamins and individual amino acids (Elliot et al., 1954). Storage of processed and irradiated shrimp samples up to 3 months (Table 4) did not affect protein quality.

Several attempts have been made to study possible genetic effects of irradiated foods using isolated cell cultures or lower organisms. Results obtained with *Drosophila melanogaster* are conflicting. It was observed that fecundity, survival or longevity was not affected when reared through seven generations on irradiated (35–55 Krad) banana (Hassan et al., 1967). But, small differences in growth, egg hatchability and longevity of this fruit fly raised on irradiated diet has been reported (Swaminathan et al., 1963). Marginal increase in sex-linked recessive lethal mutations associated with irradiated (0.15–3 Mrad) foods and food components have been noticed (Parkash, 1965; Rinehart and Ratty, 1965). This may be attributed to changes in/or partial destruction of some essential dietary components (Rinehart and Ratty, 1967) by radiation treatment. However, Chopra (1965) and Reddi et al. (1965) failed to confirm these observations under identical conditions.

A few studies have also been made with insects reared for several generations on irradiated diets. Behavior and fecundity of two species of grain weevils, *Calandra granaria* L. and *Calandra oryzae* L., were not affected when reared on irradiated (50 Krad) wheat (Cornwell and Burson, 1958). Another stored-products insect *Plodia interpunctella*, fed on irradiated (up to 4.3 Mrad) wheat for nine generations, exhibited no significant effects on numbers of progeny and sex ratio (Brower et al., 1971). However, 32% decrease in fecundity was observed when reared on pistachio nuts (Seuge et al., 1971), possibly due to toxic substances formed, which interfere with insect nutrition and reproduction. In the present studies, no deleterious effects on the growth response of *Tetrahymena* was observed (Table 5) when they were transferred at least four times to fresh media containing irradiated foods.

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SELECTED FUNCTIONAL PROPERTIES OF SUNFLOWER MEAL (Helianthus annuus)

INTRODUCTION

DURING the last two decades, oilseeds have attracted great interest as a potential source of protein for human consumption (Gheyasuddin et al., 1970). This interest is due to serious protein deficiencies in parts of the world and to increasing costs of many food ingredients from animal sources (Sosulski and McCleary, 1972; Johnson, 1970). Although used widely in parts of the world, sunflower is just now becoming a crop of accepted economic value in the United States. The oil is utilized in increasing quantities for margarines and other edible oil products because of its high quality and ease of refining. However, the residual defatted meal is not yet used in human foods due to a lack of basic knowledge concerning its functional properties. Sunflower meal, traditionally fed to animals, contains approximately 50% protein of high biological quality. Russo estimated that the cost per pound of protein to be as low as 8–12 cents in 1969.

The primary criterion for foods designed to alleviate hunger is maximum nutrition at minimum cost. However, foods designed to compete on more sophisticated markets must possess functional characteristics, aesthetic quality and moderate cost in comparison to conventional products (Stroz, 1969). The most important characteristics necessary to a finished food product acceptable to consumers are the functional properties of the ingredients in that food system (Johnson, 1970). Oilseed protein and isolates have been used in emulsions in the meat industry for many years (Saffle, 1968). They are utilized to improve color, flavor, oil emulsification, moisture retention, as well as overall cost reduction (Wilson, 1960).

The storage quality and stability of foods is related to moisture content. Food products exhibit maximum stability within an optimum range. The potential for incorporation of protein is directly related to water retention. Bread can be fortified with proteins possessing good water binding qualities as an aid to improving freshness (Hammonds and Call, 1972). According to Breuer and Kennerley (1971), water retention of proteins is related to the polar groups of the proteins and is approximated by the sum of the water-binding capacity of the component amino acids.

Oilseed proteins have been used as aerating agents in whipped toppings and frozen desserts (Wolf, 1970). They are also used in confections such as nougats and marshmallow, to partially replace egg whites in cake formulations and to impart certain desirable charactertistics to cake and cookie icings (Johnson, 1970).

In order to investigate the functional versatility of sunflower, preliminary testing of defatted sunflower meal was instigated using soybean meal as a reference. Sunflower meal compared favorably with, or exceeded, soybean meal in emulsification, water absorption and retention and whipping. These desirable characteristics would be valuable in formulating acceptable enriched food products from sunflower meals.

MATERIALS & METHODS

Sample materials

Defatted sunflower meal was prepared from five varieties of sunflower seed. These are representative of the commercial types grown in the United States and were supplied by the USDA Oilseed & Industrial Crop Research Branch, ARS, College Station, Texas. These varieties were Mingren, Peredovik, Greystripe, Arrowhead and Krasnodarets and were grown at College Station, Texas in 1970. Samples were dehulled in a Waring Blendor equipped with blades covered by rubber tubing. Blade speed was rheostatically controlled. Optimum speed was maintained to allow only cracking of the hulls without damage to the kernels. Hulls were removed by air separation. The dehulled seeds were then coarsely ground in a peanut butter mill.

Extraction of the fat was accomplished by percolating hexane through the ground material until the oil content of the meal was below 1.5% as determined by a Newport Nuclear Magnetic Resonance Analyzer, Magnet Type 10. The meal was air dried for 72 hr at 21°C and then milled to pass a 40 mesh screen and stored in a desiccator at 5°C.

Emulsion

Suspensions of 6% meal were prepared by mixing sunflower meal of the Peredovik variety and citrate-phosphate buffer. The pH was adjusted with 1N NaOH and 1N HCl to the desired level. This suspension was mixed in a Waring Blendor and oil was added. The experiment was completed at pH levels of 5.2, 7.0 and 10.8; mixing speeds of 4500, 6500 and 9000 rpm; and oil addition at the rate of 30, 45 and 65 ml/min. All rate of oil addition tests were conducted while the suspension was mixed at 4500 rpm. All speed of mixing experiments were completed with a rate of oil addition of 45 ml/min only.

Capacity of the emulsion was measured by a sudden drop in viscosity indicated by an increase in blade speed and a noticeable change in sound.

Water adsorption

Samples of each of the five varieties of sunflower meal were placed in vacuum desiccators kept at 5 and 25°C. Four different relative humidities were maintained ranging from 30–90%. Salt solutions were prepared to produce specified relative humidities as presented in Table 1. The relative humidity inside the desiccator was constantly monitored by a relative humidity indicator.

Moisture adsorption and retention were measured by determining the Equilibrium Moisture Content (EMC) at a specified Equilibrium Relative Humidity (Mellon et al., 1947). For EMC's, the samples were weighed after 36 and 48 hr in the desiccator or until there were no further changes in weight. Dry weights were obtained after drying at 110°C overnight. EMC was calculated by the following equation:

$$EMC = \frac{\text{wt of equilibrated sample} - \text{wt of oven-dried sample}}{\text{wt of oven-dried sample}}$$

Whipping

Suspensions of sunflower meal were created by dispersing an appropriate amount of meal of the Peredovik variety in 100 ml of distilled water and adjusting the pH with 1N NaOH and 1N HCl. This slurry was then brought to the desired temperature by means of a hot plate or ice bath. The suspension was transferred to a mixing bowl and whipped for 6 min at ambient temperatures with a Sunbeam Mixmaster set at the speed of 12 on the calibration scale.

Volume immediately after whipping was measured in a volumetric cylinder and reported as total ml foam. The amount of liquid released by the foam in 30 min and 2 hr was considered an indication of foam stability and was reported as ml released liquid.

The optimum conditions defined by the variables temperature, concentration and pH were determined using Response Surface Methodology. Using these optimum conditions as a basis, investigations on the sunflower meal foams were then carried out to determine individual effects of pH, temperature, meal concentration, whipping time and additives. The additives chosen were sucrose, sodium chloride, sodium phosphate tribasic, sodium hexametaphosphate and potassium bitartrate. All experiments were completed in duplicate with good agreement noted.

RESULTS & DISCUSSION

Emulsion capacity

Many factors have significant influence on the emulsification capacity of oilseed meals, including solution pH, rate of oil addition to the solution and mixing velocity.

pH. The influence of pH on emulsion capacity is shown in Figure 1. Highest capacity was obtained at a pH near 7. It has been suggested that pH influences the emulsification capacity in an indirect manner by affecting the solubility of the protein. Only the soluble protein fraction functions as an emulsifier (Swift et al., 1969). Sunflower protein solubilities were minimal at the low pH values characterizing the isoelectric point. Consequently, the emulsion capacity was not good in this range. Also, the addition of salts necessary to adjust pH and changed ionic strength have an effect on emulsification capacity (Cater and Lawhon, 1974).

Speed of mixing. Effects of mixing velocity are shown in Figure 1. Higher mixer speeds decreased the emulsifying capacity of the defatted meal. Emulsion viscosity also increased with increased mixer speeds. Becher (1957) has shown that increased oil-water emulsion viscosities with increasing rate of shear are associated with decreased oil particle size.

Rate of oil addition. Increasing the rate of oil addition increased the emulsification capacity of the meal (Fig. 1) but above a certain mixing speed the emulsification capacity dropped again. Swift et al. (1961) found the same phenomenon in meat protein. It was suggested that the rate at which protein membranes are formed is almost instantaneous at high oil addition rates.

Water adsorption and retention

Varietal evaluations. Equilibrium Moisture Contents (EMC) of five varieties of sunflower meal were obtained at specified equilibrium relative humidities as shown in Figure 2. On the average, Krasnodarets had the highest water retention and Mingren the lowest. Little difference was noted in the moisture content of samples between relative humidities of 10 and 70%. Water adsorption rose rapidly above 80% relative humidity.

The basis for the differences in water retention by different varieties is uncertain. Differences in the carbohydrate fractions of the meals might be the reason for the observed variations (Kilara and Humbert, 1972). Berlin et al. (1968) suggested that salts and proteins are the principal components responsible for water adsorption in an oilseed meal. The total sugar content and the protein content of the five varieties of sunflower meal investigated here are presented in Table 2. Protein content was highly correlated to water adsorption. Temperature had no noticeable effect on water adsorption.

Moisture sorption isotherm. The moisture sorption isotherm (MSI) for sunflower meal is plotted in Figure 3. The whole sorption isotherm is divided into three parts called localized isotherms (L.I.) where inflection points of the curves occur. Optimum product stability generally appears to be associated with L.I.-II. At lower moisture levels within the L.I.-I, free radicals induced by irradiation or oxygen absorption may potentiate rancidity. Local Isotherm III appears to define the region in which free water has a dominant influence on food product stability. Unfavorable enzyme reactions are accelerated within L.I.-III (Rockland, 1969).

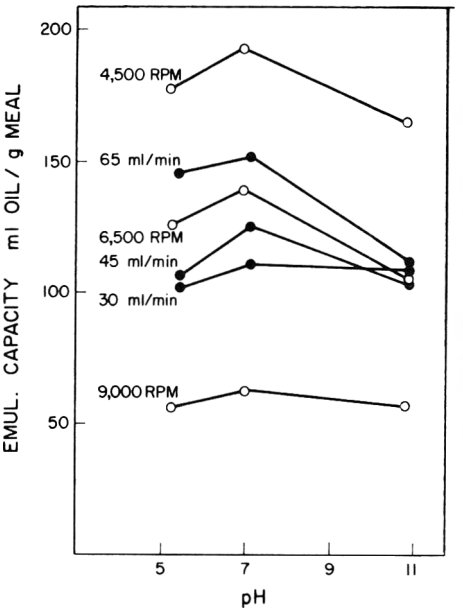

Fig. 1—Effect of pH, speed of mixing and rate of oil addition on the emulsification capacity of sunflower meal.

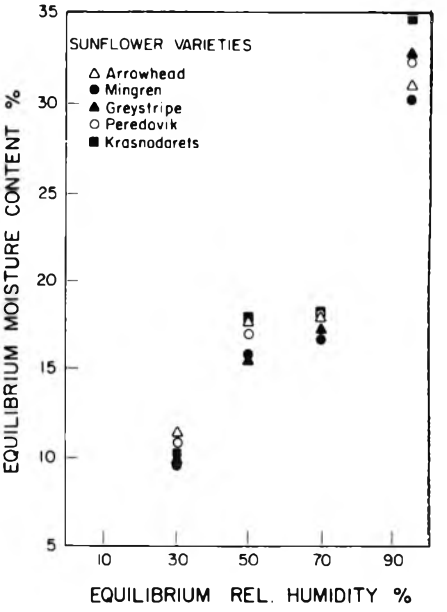

Fig. 2—Water adsorption of sunflower meals.

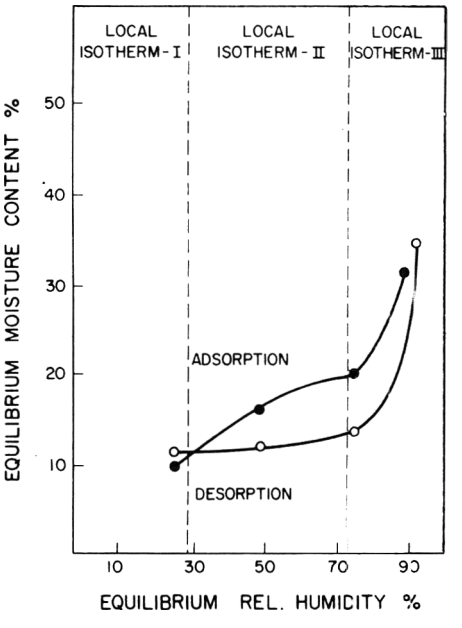

Fig. 3—Sorption isotherm for Arrowhead variety sunflower meal at 25°C.

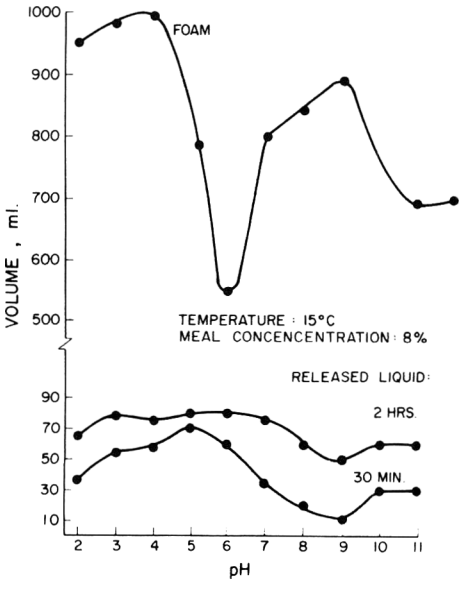

Fig. 4—Volume and stability of sunflower meal foams as affected by pH.

The curve is in agreement with Rockland's (1969) MSI for nut kernels with high portions of protein and soluble solids and small amounts of starch and cellulose. Compared with the MSI of other food products, this curve has the minimum moisture, equilibrium relative humidity range at L.I.-H.

Whippability. The optimum pH, temperature and meal concentration for production of voluminous, stable, fine-textured foams was pinpointed by Response Surface Methodology. pH 9.00, 8% meal concentration and 15°C were these optimum conditions.

Effect of pH. 8% meal dispersions at various pH values were chilled to 15°C and then whipped for 6 min. Figure 4 shows the effect of pH on foam expansion and released liquid. The desired volume increase was obtained below pH 4 and above pH 8. The poorest foams were obtained in the isoelectric region, near pH 5. This is in agreement with the theory that a protein must be soluble in order to foam (Lawhon and Cater, 1971; Eldridge et al., 1963). Stable foams were obtained above pH 8. Maximum leakage, occurring in the isoelectric region, was 80%. The deviation from a theoretical value of 100% was attributed to the amount of foam adhering to the sides of the volumetric cylinders (Eldridge et al., 1963). Even though the volume of foam is greater at pH 4, the optimum pH should be considered 9 since the stability is better there than at pH 4.

The effect of pH on the foam color, volume and texture can be seen in Figure 5. Sunflower meal contains a chromogen, chlorogenic acid, which is pH activated. The color changes and darkens as pH increases. As illustrated in Figure 5, foams formed at a low pH, around 5, had acceptable color but low volume and coarse texture. Those formed at pH 9 had green color but optimum volume and fine texture. At pH 11, the color darkened further, volume declined and texture coarsened.

The effect of pH on foam stability can also be seen in Figure 5. Foams pictured here were produced 6 min apart and photographed after 60 min. The lack of stability of low pH products is quite apparent.

Effect of temperature. Eldridge et al (1963) and others have shown the necessity of heating soybean protein to produce a stable foam. In sunflower meal it was found in this study that this heating is not desirable. As shown in Figure 6, elevated temperature affects foam volume. Volume decreases above 55°C and the decrease becomes extensive above 65°C. This is evidently due to denaturation of protein and subsequent loss of sol-

Table 1—Relative humidity of equilibrated air over saturated salt solutions at different temperatures

Salt	RH%	Temp °C
Potassium acetate	26	5
	28	25
Calcium chloride	51	5
	48	25
Sodium chloride	71	5
	73	25
Potassium nitrate	92	5
Potassium sulfate	92	25

Table 2—Total sugar and protein content of defatted sunflower kernels

Variety	Total sugar (% dry wt)	Protein (% dry wt)
Peredovik	4.99	58.44
Krasnodarets	4.41	61.27
Mingren	4.91	56.59
Arrowhead	4.69	56.59
Greystripe	6.28	–

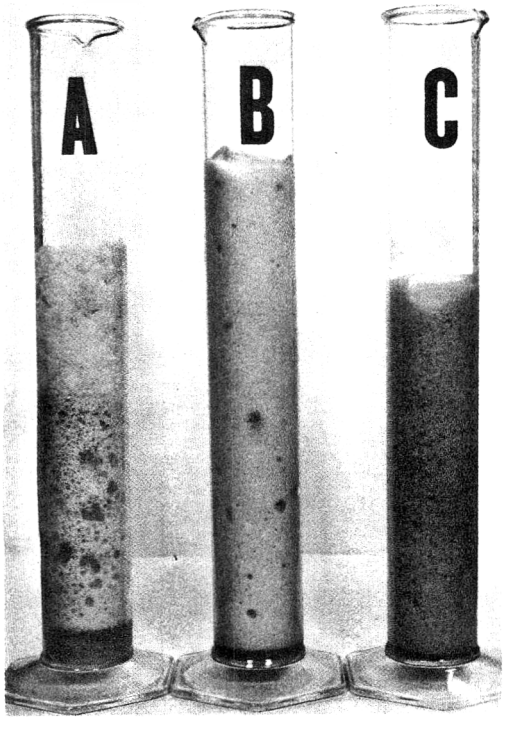

Fig. 5—Sunflower meal foams as affected by pH: (A) pH 5; (B) pH 9; (C) pH 11.

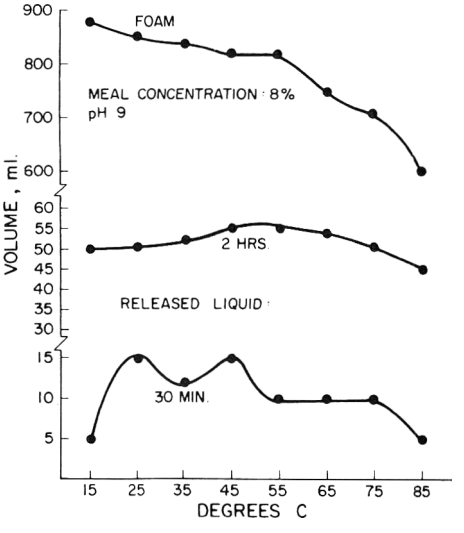

Fig. 6—Volume and stability of sunflower meal foams as affected by temperature.

ubility (Yasumatsu et al., 1972). Temperature had very little effect upon foam stability.

Meal concentration. Figure 7 illustrates the effect of meal concentration on volume and stability of sunflower meal foams at pH 9 and 15°C. Low meal concentrations produced a foam with poor volume and very poor stability. As the meal concentration was increased, foam volume increased and foam stability improved up to 8% concentration. Above 8%, the volume declined.

Effect of whipping time. Figure 8 illustrates the effect of whipping time on foam volume and foam stability at pH 9. 8% dispersions, cooled to 15°C, were whipped for various periods of time. Volume increase and stability were determined. The volume increased markedly up to 12 min, then began a slow decline. Foam stability gradually approached a maximum between 7 and 10 min, then decreased slowly with continued whipping. Similar effects of continued whipping on foam stability have been reported earlier for soybean foams (Eldridge et al., 1963) and for gliadin foams (McDonald and Pence, 1961). Sunflower meal foams would not be subject to degradation due to overbeating in a commercial operation.

Effect of additives. In use, foaming products usually contain salts, flavoring, sugar and other additives (Eldridge et al., 1963). Therefore, 8% dispersions were adjusted to pH 9 and chilled to 15°C. After 1 min whipping, adjuncts, which approximate common formulations, were added and whipping was completed (Table 3). Foam volume and released liquid were measured and reported in Figure 9. The addition of sucrose caused a decrease in volume but increased stability at 30 min. Stability decreased slightly at 2 hr. The addition of 5% NaCl caused a marked increase in overrun but had little effect on stability. Very little effect was noted when either of the phosphates was added. Potassium bitartrate affected a color change from green to white. This was due to a lowering of pH from 9 to 5. Also noted was a drastic reduction in volume and a marked decrease in stability. Of great interest was the fact that potassium bitartrate added in combination with sucrose produced a foam with increased volume, extreme stability and a bright white color.

Table 3—Adjuncts used in foams from sunflower meal

Additive	Concentration (g/100 ml sol)[a]
Sucrose	60
Sodium chloride	5.0
Sodium phosphate, tribasic	0.024
Sodium hexametaphosphate	0.047
Potassium bitartrate	1.3
Potassium bitartrate plus sucrose	1.3 + 60

[a] Eldridge et al. (1963)

CONCLUSIONS

SUNFLOWER MEAL has great potential for incorporation into human food products, not only as a protein supplement in diets of undernourished people, but also as a functional agent in foods designed for markets in developed countries. Sunflower meal exhibits excellent functional properties. The most promising properties, emulsion capacity, water adsorption and retention and aeration properties, were examined in this study.

Optimum emulsion capacity is at pH 7. Lower speed of mixing and fast rate of oil addition increase emulsion capacity. There are varietal differences in the water adsorption capacity of sunflower meals. This is attributed to varying protein concentrations. Optimum foam production and foam stability are achieved at pH 9, meal concentration 8%, temperature 15°C and a whipping time of 12 min. The addition of sucrose and potassium bitartrate to the foam during whipping produces a bright white foam with good volume and excellent stability.

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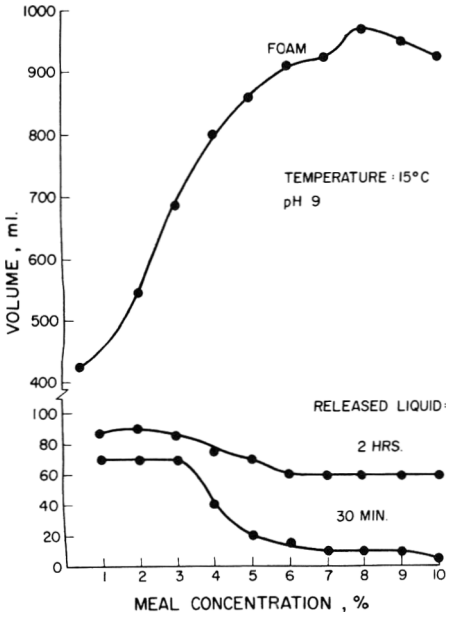

Fig. 7—Volume and stability of sunflower meal foams as affected by concentration.

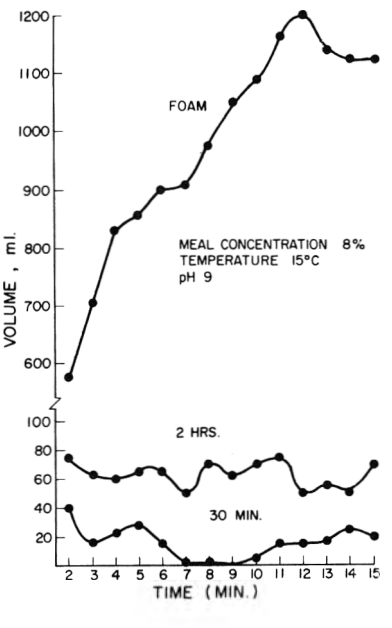

Fig. 8—Volume and stability of sunflower meal foams as affected by whipping time.

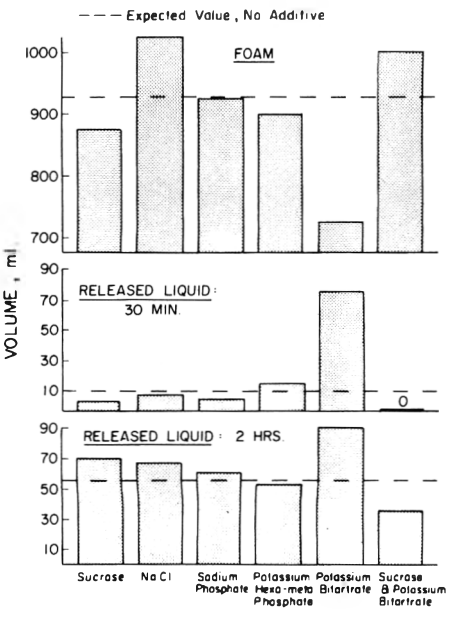

Fig. 9—Volume and stability of sunflower meal foams as affected by additives.

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PROTEIN QUALITY AND SUPPLEMENTARY VALUE OF COTTONSEED FLOUR

INTRODUCTION

ONE HUNDRED MILLION tons of protein are needed annually for human consumption and perhaps twice as much for feeding to livestock. Oilseeds, such as cottonseed, offer a partial solution to this need (Bressani, 1965; Lawhon et al., 1972). In 1970, approximately 24 million tons of cottonseed were produced in the world (Anonymous, 1970). Thus, cottonseed is a readily available source of protein both in domestic and world markets. However, the toxic quality of gossypol in regular glanded cottonseed has been a handicap to its usage by nonruminating animals (Conkerton and Frampton, 1959; Sharma et al., 1966). Cotton breeders have developed a glandless cottonseed which is currently available in limited quantities (Harper and Smith, 1968; Decossas et al., 1968). Furthermore, Pons and Eaves (1967) and Gastrock (1968) have reported a liquid cyclone process (LCP) to remove gossypol from regular glanded cottonseed which yields a low-gossypol cottonseed flour. A LCP plant has been installed at the Plains Cooperative Oil Mill in Lubbock, Texas, and the anticipated daily yield is 20–25 tons of a 67–70% protein cottonseed flour of a quality suitable for human consumption (Ziemba, 1972).

Four food categories of paramount need for supplementary protein are: (1) various dairy products; (2) cereal-based products; (3) pet foods; and (4) meat formulations. To meet these demands, the supplement must promote certain functional properties of the food and improve the nutritional value of the final product (Melnychyn, 1972).

With the impending availability of LCP cottonseed flour for human consumption, investigations were conducted to determine the quality of the proteins in cottonseed by amino acid analyses and net protein utilization (NPU). Rooney et al. (1972), Matthews et al. (1970) and Tsen et al. (1971) used various levels of cottonseed flour substituting wheat flour in yeast breads to illustrate functional properties. In the present study, 18.8% cottonseed flour was substituted for wheat flour in a yeast bread to demonstrate that a significant nutritional contribution can be made to a staple food through protein fortification.

EXPERIMENTAL

Materials

Samples of LCP cottonseed flour provided by the USDA Southern Regional Research Center, ARS, New Orleans, La., glandless cottonseed flour supplied by Texas A&M University Oilseed Products Research Center, and glanded cottonseed flour supplied by the Plains Cooperative Oil Mill, Lubbock, Texas, were compared with hexane-extracted soybean oil meal and casein.

Chemical and biological tests

Protein contents (N × 6.25) of the samples were determined by the Macro-Kjeldahl procedure (AOAC, 1970). For amino acid determination, samples containing 7 ± 0.5 mg protein were hydrolyzed with 2 ml 12N hydrochloric acid under vacuum in an air oven at 105°C for 22 hr. The hydrolysates were filtered, evaporated to dryness, and made to 5 ml with a pH 2.2 sodium citrate buffer. Amino acid composition of the samples was determined by ion-exchange column chromatography using a Model 116 Beckman amino acid analyzer (Moore and Stein, 1954). As cysteine and tryptophan were destroyed by acid hydrolysis, their values were not reported.

The moisture contents of the yeast breads were determined by drying the samples in an air oven at 110°C to a constant weight (AOAC, 1970). The total lipid contents were determined by the method of Folch et al. (1957).

The biological values of the different proteins were determined by animal growth experiments using 25-day old male weanling rats of Sprague-Dawley strain. Six animals were randomly assigned to each diet. They were placed in individual, raised screen-bottomed cages in an air-conditioned room and given food and water ad libitum for 10 days. An otherwise adequate but protein-free diet consisting of

Table 1—Composition of yeast breads

	Variations		
Ingredients	Glandless cottonseed flour (g)	LCP cottonseed flour (g)	Wheat flour (g)
Active dry yeast	9.5	9.5	9.5
Water	226.9	226.9	226.9
Cottonseed flour	71.4	71.4	—
Wheat flour	308.5	308.5	379.9
Sugar	20.3	20.3	20.3
Salt	10.3	10.3	10.3
Corn oil	25.4	25.4	25.4

Table 2—Moisture, protein and lipid content of baked yeast breads[a]

Variations	Moisture (%)	Protein (%)	Lipid (%)
Glandless flour bread	25.9	21.13	7.14
LCP flour bread	23.5	19.06	5.73
Wheat flour bread	22.2	10.48	8.21

[a] See Table 1 for composition

Table 3—FAO amino acid pattern[a] and eight essential amino acid contents (g/100g protein) of samples

Amino acid	FAO pattern	Cottonseed flours			Soybean meal	Casein	Yeast breads[b]					
							Wheat		LCP cottonseed		Glandless cottonseed	
		LCP	Glanded	Glandless			Dough	Baked	Dough	Baked	Dough	Baked
Lysine	4.2	5.4	5.6	4.4	8.6	7.7	3.0	2.5	4.3	3.8	4.3	3.8
Threonine	2.8	4.1	4.4	4.7	4.9	4.9	3.4	3.4	3.5	3.6	3.8	3.4
Valine	4.2	4.8	5.1	5.1	4.6	5.0	4.6	4.0	5.0	5.0	4.9	5.4
Methionine	2.2	1.8	2.2	2.0	1.8	3.5	1.9	1.7	2.1	2.0	2.1	2.3
Isoleucine	4.2	3.6	5.0	3.6	4.5	4.1	4.0	3.9	4.0	4.0	4.0	4.2
Leucine	4.8	7.3	8.8	7.6	9.2	10.2	8.4	8.3	8.7	8.3	8.3	8.6
Tyrosine	2.8	3.9	3.6	3.9	4.3	6.3	3.1	2.9	3.3	3.6	3.7	3.6
Phenylalanine	2.8	6.8	6.8	6.6	6.1	5.7	5.8	6.3	7.0	6.7	6.4	6.4

[a] Food & Agriculture Organization (1965)
[b] See Table 1 for composition

Table 4—Growth data of young rats fed diets containing cottonseed flours, soybean oil meal or casein

Protein source	Level of protein					
	10%			20%		
	Food intake (g)	Wt gain (g)	NPU[a] (%)	Food intake (g)	Wt gain (g)	NPU (%)
Glanded cottonseed	all dead by the 5th day					
Glandless cottonseed	99.6	31.6	58.0	98.0	51.9	59.3
LCP cottonseed	105.6	32.7	57.2	84.2	51.4	56.4
Soybean oil meal	81.4	28.2	65.8	99.5	58.3	63.2
Casein	87.3	32.4	78.6	94.1	65.8	69.1

[a] Net protein utilization (NPU) = $\frac{\text{Nitrogen (n) gain} \times 100}{\text{N intake}}$

83% cornstarch, 10% corn oil, 4% salts (Jones and Foster, 1942), 2% alphacel and 1% vitamin mixture (Yang et al., 1959) was used as the basal diet. For the experimental diets, 10 and 20% protein supplied by cottonseed flour, soybean and casein were added to the basal diet at the expense of cornstarch. At the termination of the 10-day feeding period, the animals were sacrificed and autoclaved for determination of carcass nitrogen (Sarett and Snipper, 1954). The net protein utilization (NPU) was calculated by the formula of Miller and Bender (1955).

Supplementation of yeast breads with cottonseed flour

Glandless and LCP cottonseed flours were used to prepare yeast breads following a family recipe for cottonseed flour published by USDA (Anonymous, 1969). The breads were baked in an All-Purpose Toastmaster oven at 163°C for 30 min. Wheat flour bread varied only in the substitution of all-purpose enriched wheat flour for cottonseed flour. As described in the family recipe, the time and speed of mixing and kneading for cottonseed flour breads have to be reduced as cottonseed flour dough is sensitive to overmanipulation. The fermentation and proofing times have to be decreased, the baking temperature lowered, and the baking time increased in comparison to the procedures used for wheat flour yeast breads.

RESULTS & DISCUSSION

CRUDE PROTEIN contents of "as is" LCP cottonseed flour, glanded cottonseed flour, glandless cottonsed flour, soybean oil meal and casein were 71.0, 59.9, 57.3, 43.3, and 86.8% respectively. The compositions of the yeast breads are shown in Table 1.

Cottonseed bread is heavier, more compact, rougher textured and has a color comparable to that of a whole wheat bread. Matthews et al. (1970) also found this to be true in baking with oilseed flours when replacements for 25% of wheat flour were made. Rooney et al. (1972) reported that cottonseed flour differs markedly in functional characteristics when mixing and baking into a yeast bread.

The moisture, protein and lipid contents of the baked yeast breads are shown in Table 2. The protein contents of the glandless and LCP cottonseed yeast breads were substantially higher than that of the wheat bread while their moisture and lipid contents were not markedly different.

Amino acid analyses (Table 3) indicate that the lysine content of the cottonseed protein after the removal of gossypol was slightly decreased but not below the value of Food & Agricultural Organization (FAO) amino acid pattern (1965) or the value of the glandless cottonseed flour. The lysine values for all cottonseed flour samples were lower than those of soybean oil meal or casein. Yeast breads containing LCP or glandless cottonseed flour, either at dough stage or as a baked product, contained more lysine than the comparable wheat bread samples. The lysine contents of all baked yeast breads were lower than that of the FAO pattern. Although the threonine contents of the yeast bread were lower than those of the cottonseed flours and other samples, all the samples tested in the present study contained more threonine than the FAO pattern.

All cottonseed flours and yeast breads contained adequate quantities of valine, leucine, tyrosine and phenylalanine as compared with FAO values. Some cottonseed flours and yeast breads, however, were slightly low in methionine and isoleucine.

Comparison of the amino acid contents of the breads (Table 3) illustrates the value of supplementing wheat flour with cottonseed flour. The lysine contents of both the dough and baked cottonseed flour breads were substantially higher than that of the comparable wheat flour bread samples. In each instance baking decreased the lysine content of wheat, LCP and glandless flour breads 15, 12 and 14%, respectively. Clark et al. (1959) reported that baking destroyed 9% of the lysine in a wheat flour bread. As shown in Table 3, other amino acid values varied slightly after baking.

There were no substantial differences in the weight gains between rats fed the glandless or LCP cottonseed flours at either 10 or 20% protein level (Table 4).

Table 5–Growth data of rats fed diets containing cottonseed for wheat flour breads as the sole article[a] for 10 days

Bread diets	Food intake (g)	Protein intake (g)	Wt gain (g)	Food efficiency[b]
Glandless cottonseed flour	111.9	23.6	46.8	0.42
LCP cottonseed flour	103.8	19.8	38.6	0.37
Wheat flour	66.5	7.2	4.4	0.07

[a] See Table 1 for composition
[b] Weight gain/food intake

Table 6–Growth data of rats fed diets containing 10% protein provided by cottonseed or wheat flour breads for 10 days

Bread	Food intake (g)	Wt gain (g)	NPU[a]
Glandless cottonseed flour	88.4	9.1	43.11
LCP cottonseed flour	77.2	14.8	47.31
Wheat flour	67.4	5.6	34.01

[a] See Table 4

Rats receiving a diet containing 10% protein from glanded cottonseed flour died by the 5th day of the experiment. At the 10% protein level, the net protein utilization (NPU) of soybean diet was 12–13% higher than the cottonseed flour diet. The NPU of soybean diet at 20% protein level was 6–10% higher than the values of the cottonseed flour diets. The casein diet was 26–28% higher at the 10% protein level and 4–14% higher at the 20% protein level as compared with the comparable cottonseed flour diets. Thus, greater difference was apparent at the 10% protein levels than at the 20% protein levels. About 30 yr ago, Grau (1946) showed that lysine was the first limiting amino acid and methionine the second limiting amino acid in the proteins of cottonseed flour. Later studies of Fisher (1965) indicated that in addition to lysine and methionine, leucine, threonine and isoleucine were also limiting for optimum growth of chicks receiving cottonseed meal.

As shown in Table 5, substantial weight differences occurred among the rats given the diets containing the wheat flour bread as the sole article for 10 days. The differences were probably due to the lower protein content of the wheat flour bread (Table 2). No substantial difference in weight gains occurred in rats receiving glandless or LCP flour breads.

The weight gains and NPU values of the animals fed the two cottonseed bread diets at the 10% protein level were substantially higher than those fed the wheat bread diet (Table 6). The superiority of the cottonseed flour breads is probably due to the higher contents of lysine and methionine as shown in Table 3. It is apparent that the substitution of 18.8% cottonseed flour for wheat flour substantially improved the nutritional value of the bread.

In summary, chemical analyses and biological studies of cottonseed flours and yeast breads containing cottonseed flours have demonstrated that the proteins from cottonseed flours can make a significant nutritional contribution to our diet. Cottonseed flour should be useful in many food items and protein-rich cottonseed products are seen as having a particularly great potential in areas of the world with problems of undernourishment or starvation. Palatable, nutritious and acceptable food products can be made by incorporating varying levels of cottonseed flour into many food items. Further studies, however, are needed to evaluate the palatability and the acceptability of cottonseed flour yeast bread and breads made from combinations of cottonseed flour, sorghum, soybean, triticale or many other food items.

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AN ENZYMATIC-CHEMICAL METHOD FOR EXTRACTION OF COTTONSEED PROTEIN

INTRODUCTION

EFFICIENT PRODUCTION of high quality cottonseed proteins has been dependent upon production of defatted flours in which the protein bodies are not glued together by denatured cytoplasmic proteins but rather are free to separate from other cellular particulates (Martinez et al., 1970). If these criteria are met, cottonseed protein isolates can be produced by numerous means including (a) a two-step extraction procedure involving water and dilute alkali (Berardi et al., 1969); (b) ethanol extraction of undenatured flours (Berardi, 1968, in Martinez et al., 1970); and (c) aqueous (pH 6.3–6.8, 0.008M $CaCl_2$) extraction of undenatured flours (Martinez, 1969, in Martinez et al., 1970). No literature describing efficient means for extraction of protein from partially denatured cottonseed flours or meals was found.

Recent experimental studies have suggested that the use of proteolytic enzymes to partially digest coconut meal prior to chemical extraction with dilute alkali will increase the efficiency of protein extraction from the meal (Molina and Lachance, 1973). Arzu et al. (1972) have shown that cottonseed proteins can be hydrolyzed by a number of proteolytic enzymes. The current series of experiments were undertaken to ascertain the effectiveness of a combined enzymatic-chemical technique for extraction of protein from cottonseed meal.

Specific experiments were undertaken to determine (a) the relative efficiency of an enzymatic-chemical method vs. the two-step chemical extraction method of Berardi et al. (1969); (b) the comparative effectiveness of three proteolytic enzymes: trypsin, ficin and papain; (c) the relationship of the pH of the enzyme buffer to the efficiency of protein extraction; (d) the relationship between the amount of enzyme used and the amount of protein solubilized; and (e) the efficiency of the enzymatic-chemical method for extraction of protein from further heat-treated cottonseed meal.

MATERIALS & METHODS

THREE SAMPLES of screw-expressed cottonseed meal (41% protein) were purchased at a local feed mill. The samples were held at room temperature in sealed plastic bags until experimentation.

Chemical extraction technique

25g of cottonseed meal were suspended in 100 ml of distilled water at 50 ± 1°C and stirred for 60 min. This suspension was filtered through cotton organdy. The filtrate (enzyme control fraction) was held for Kjeldahl nitrogen analysis. The filter cake was suspended in 100 ml of 0.075% NaOH and stirred at 60 ± 1°C for 60 min (temperatures > 60°C did not increase the efficiency of extraction). This suspension was also filtered through cotton organdy. Both the filtrate (NaOH control fraction) and filter cake (residue control fraction) were held for Kjeldahl nitrogen analysis. Berardi et al. (1969) used a 15:1 liquid/meal ratio for both steps of a selective protein extraction. These data reflect a lower ratio of 4:1. A conversion fraction of 6.25 was used to convert N to apparent protein.

Enzymatic-chemical extraction technique

25g of cottonseed meal and 0.10g of enzyme were stirred with 100 ml of buffer (0.02M sodium phosphate) at 50 ± 1.0°C for 60 min. Literature values for optimum pH activity (Molina and Lachance, 1973) were utilized as follows: papain–Nutritional Biochemicals Co. purified concentrate (pH 7.5); trypsin–Nutritional Biochemicals Co. 1-300 hog pancreas (pH 8.2); ficin–Nutritional Biochemicals Co. partially purified powder (pH 5.8). This mixture was filtered through cotton organdy and the filtrate (enzyme fraction) held for Kjeldahl nitrogen analysis. The filter cake was suspended in 100 ml of 0.075% NaOH and stirred for 60 min at 60 ± 1°C. This suspension was also filtered through cotton organdy and both the filtrate (NaOH fraction) and filter cake (residue fraction) held for Kjeldahl analysis.

Effect of pH of enzyme incubation on protein extraction

The enzymatic-chemical technique was used to measure the amount of protein extracted but the pH of the enzyme incubation buffer was varied. Phosphate buffers (0.02M) with pH's from 3.0–11.0 in 0.5 increments were employed.

Effect of enzyme concentration on protein extraction

The enzymatic-chemical technique described above was used to quantify protein extraction and the amount of trypsin was varied. The amounts of enzyme used were: 0g, 0.0005g, 0.001g, 0.005g, 0.01g, 0.02g, 0.03g, 0.06g and 0.12g. All trypsin incubations were at pH 8.2.

Effect of further heat treatment on protein extraction

Samples of cottonseed meal were heated for 30 min at 121, 149, 176 or 204°C. The enzymatic-chemical technique described above was utilized to determine the extraction of proteins from further heat-treated samples.

RESULTS

Effectiveness of proteolytic enzyme pretreatment

Without enzyme treatment, only 15% of the cottonseed protein was extracted by a two-step chemical extraction (Fig. 1) Treatment with papain did not improve the efficiency of extraction. Ficin treatment increased the amount of protein extraction approximately 2.5 times. Trypsin treatment was most effective, causing an approximate fivefold increase in extraction with greater than 75% of protein being extracted. Because of the ineffectiveness of papain, it was not utilized in further experimentation and was replaced by bromelain (Midwestern Biochemical Corp.).

Effect of pH of enzyme incubation media on protein extraction

Trypsin treatment was more effective than ficin or bromelain at pH's from 4.0–9.5 (Fig. 2). There was finite but little variation amongst the enzyme treatments with respect to the protein content of the enzyme fraction (Fig. 3). Trypsin treatment, however, resulted in a two- to threefold increase in the protein content of the NaOH fraction relative to ficin or bromelain treatment (Fig. 4). This would suggest that trypsin may affect the cottonseed meal substrate in ways other than simple protein hydrolysis. The nature of this effect(s) is not readily apparent. Because of the markedly greater efficiency of trypsin, it alone was evaluated in further experiments.

Relationship of enzyme concentration to protein extraction

The relationship of *enzyme* concentration to protein extraction was curvilinear (Fig. 5). Treatment with trypsin concentration of 0.03g/25g meal caused greater than 75% of the protein to be extracted. At greater than 0.03g enzyme/25g meal, the curve was essentially flat and use of additional enzyme did not increase the yield of protein.

Effect of further heat processing on protein extraction

Further heat treatment caused a de-

crease in the efficiency of protein extraction by the combined enzymatic-chemical method utilizing trypsin (Fig. 6). This decrease was a linear function of temperature. Regression analysis of temperature (x) vs. enzyme fraction protein (y) yielded a correlation coefficient of 0.9043 and a regression equation defined as:

$$y = -0.0087(x) + 4.1093$$

where y is the total protein extracted from the 25g meal samples and x is the temperature in °C. A similar regression analysis of the NaOH fraction (same units) gave a correlation coefficient of 0.9853 and a regression equation of:

$$y = -0.0119(x) + 4.9054$$

Even though the decrease was linear, protein recovery from cottonseed meal treated at 204°C using the combined enzymatic-chemical method was more efficient than the two-step chemical extraction procedure for the control unheated samples.

Comment

The combined proteolytic enzyme-chemical method described here appears to have several desirable attributes with reference to extraction of cottonseed protein: (a) the method allows extraction of more protein than a normal two-step chemical extraction; (b) this improved extraction efficiency is still apparent with cottonseed meal samples held at 204°C for 30 min; (c) the activity of trypsin in this method was not greatly changed over

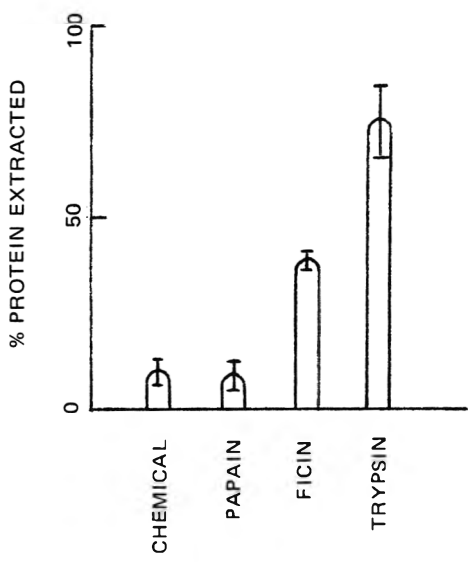

Fig. 1—Efficiency of a two-step chemical extraction technique and a combined enzymatic-chemical technique utilizing papain, ficin, or trypsin for extraction of cottonseed protein. All data bars are the mean of three replicates ± standard deviation. See text for condition of extractions.

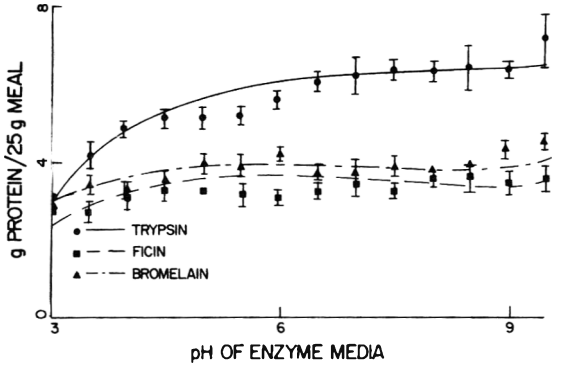

Fig. 2—Total g protein extracted from 25g cottonseed meal by means of a combined enzymatic-chemical technique. The efficiency of three enzymes is compared—trypsin, ficin, and bromelain—as a function of pH. All data points are the mean of three replicates ± standard deviation.

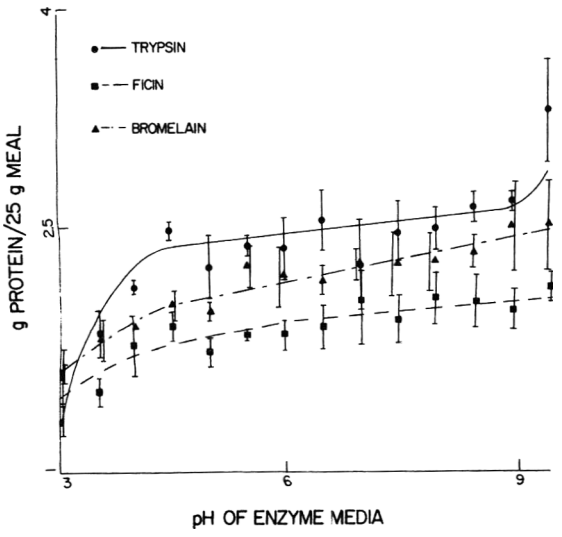

Fig. 3—Grams protein extracted in the enzyme fraction from 25g cottonseed meal by means of a combined enzymatic-chemical technique. The efficiency of three enzymes is compared—trypsin, ficin and bromelain—as a function of pH. All data points are the mean of three replicates ± standard deviation.

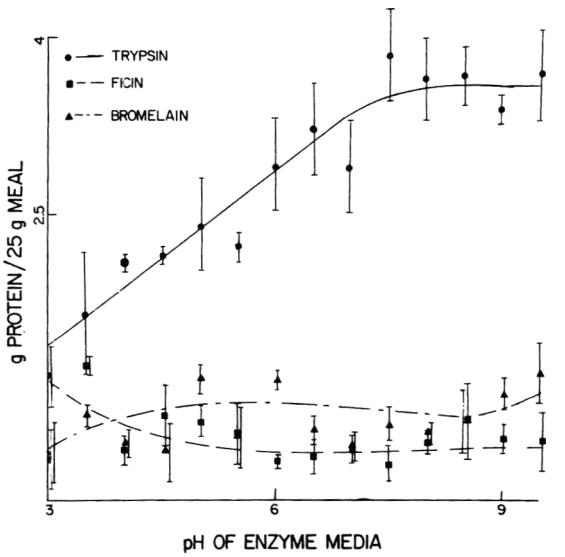

Fig. 4—Grams protein extracted in the NaOH fraction from 25g cottonseed meal by means of a combined enzymatic-chemical technique. The efficiency of three enzymes is compared—trypsin, ficin and bromelain—as a function of pH. All data points are the mean of three replicates ± standard deviation.

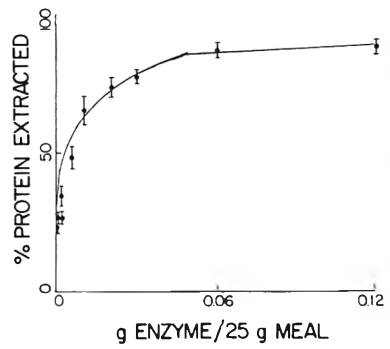

Fig. 5—The effect of trypsin concentration on extraction of cottonseed meal protein. All data points are the mean of three replicates ± standard deviation. See text for conditions of extraction.

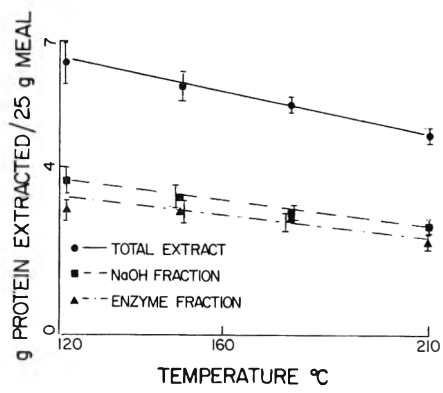

Fig. 6—Effect of further heat treatment on extractability of cottonseed meal proteins. All data points are the mean of three replicates ± standard deviation. See text for conditions of extraction.

a fairly wide range of pH's (6.5–9.5); and (d) the method works effectively with a 4:1 liquid/meal ratio as compared to a 15:1 ratio with conventional treatments (Berardi et al., 1969).

Although treatment with as little as 0.03g trypsin per 25g meal allowed extraction of greater than 75% of available protein, economic development of this process will probably require development of techniques which will utilize immobilized trypsin (Knights and Light, 1974). In addition, the functional and nutritional properties of the protein must be evaluated.

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FUNCTIONAL PROPERTIES OF THE GREAT NORTHERN BEAN (Phaseolus vulgaris) PROTEIN ISOLATE

INTRODUCTION

IN SPITE OF their relatively high cost per pound of protein, meat, eggs and dairy products are consumed in amounts approximately ten times greater than peas and beans, even though the latter are abundant in protein. The marked preference for animal proteins may be related to their unique balance of essential amino acids, especially methionine, which is the limiting amino acid in beans. A second problem associated with bean products is the gastrointestinal distress which can occur after ingestion.

The nutritive value of several beans has been investigated. Kakade and Evans (1965) described the nutritive value of navy beans (*Phaseolus vulgaris*). Unheated navy beans contain trypsin inhibitor and hemagglutinating activities which are destroyed by heating 5 min at 121°C. Autoclaved beans, when measured, had a protein efficiency ratio (PER) of 1.84, casein having a PER of 2.85. When autoclaved navy bean protein was compared to the FAO essential amino acid pattern, the bean protein was limiting only in methionine. All other essential amino acids were present in abundance.

Kakade and Evans (1964) wet fractionated navy beans and demonstrated that the hemagglutinating activity of the bean is located in an acid (pH 4.0) soluble fraction. The trypsin inhibitor activity was also found in that fraction. The majority of the bean protein is insoluble and precipitates at pH 4.0. Therefore, simple isoelectric precipitation of bean protein at pH 4.0 separates the bean protein from its antinutritional factors. Murphy (1963) has shown that the flatus factor, which causes gastrointestinal distress 5–7 hr after ingesting beans, is soluble in 60% ethanol. Murphy et al. (1964) demonstrated that a 73% protein isolate from California small white beans (*Phaseolus vulgaris*) was void of almost all of the flatulence factor and antinutritional factors.

It was the purpose of this study to investigate the functional properties of a protein isolate from Great Northern beans (*Phaseolus vulgaris*).

MATERIALS & METHODS

GREAT NORTHERN BEANS were hammer milled to yield a flour. The bean flour was soaked overnight in 2% NaCl and the residue was removed via centrifugation at 9000 × G for 30 min. The supernatant obtained was dialyzed for 48 hr against distilled water. The resulting precipitated globulins were removed by centrifugation at 9000 × G. The pH of the resulting supernatant was lowered to 3.5 with 6N HCl and the precipitated albumins were then removed via centrifugation. All fractions obtained were freeze dried and stored at 20°C until needed. Besides the albumin and globulin fraction, the two were also recombined in the proportions that exist in the bean (1.00 part albumins to 1.85 parts of globulins). The recombined proteins are designated as BPC, bean protein concentrate (Fig. 1).

Proximate analyses for protein, fat, ash and moisture were performed according to the *Official Methods of Analysis* (AOAC, 1970).

Emulsion capacity determinations were accomplished with a micro-emulsifier, using the procedure of Tsai et al. (1970) as modified by Satterlee et al. (1973). The emulsion stability test, as described by Satterlee et al. (1973), was used in this study.

The whipping properties of the bean proteins were determined with a "Kitchen Aid" blender at room temperature. A measured amount of protein (1, 3, 5 or 10g) was placed into distilled water to make a total of 100 ml of dispersion and then whipped. The amount of foam obtained was measured in a graduated cylinder. A 1% protein dispersion was used in the pH tests. The pH was adjusted to either 4, 5, 6, 7 or 8, using 1N HCl or NaOH, hourly for 4 hr prior to whipping. A 1% protein dispersion was also used in the temperature tests to determine the effect of heat on foaming. The protein dispersion was kept at the desired temperature for 5 min prior to whipping.

To determine if any residual lipids were hindering whipping, the protein isolate was washed with acetone or ethyl alcohol and then tested. Distilled water was used to remove all solvents from the protein. Volume increase and percent syneresis were calculated as follows:

$$\text{vol inc (\%)} = \frac{\text{vol after whipping (100)}}{\text{vol before whipping}}$$

$$\text{\% syneresis} = [(\text{ml released from foam after 30 min standing})/(\text{ml initial vol} - \text{ml initial vol not formed into foam})]\ (100)$$

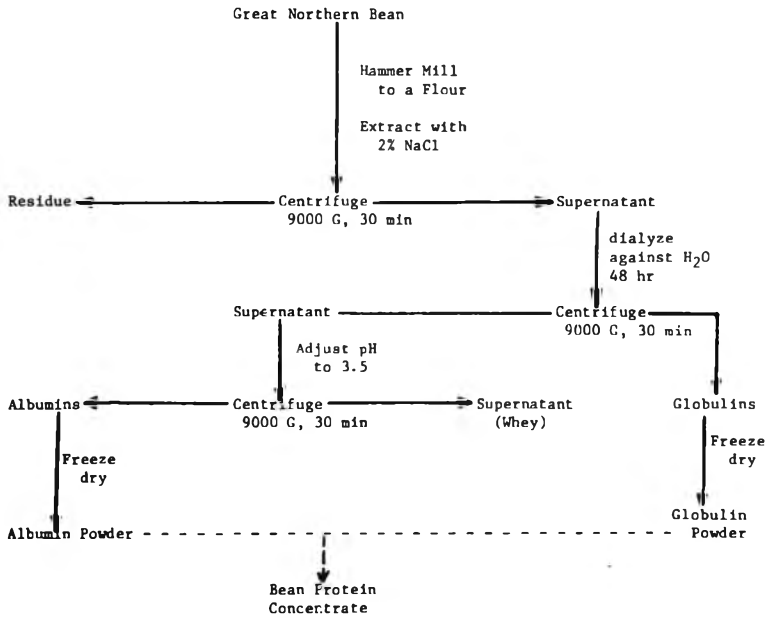

Fig. 1—Procedure used to isolate proteins from Great Northern bean.

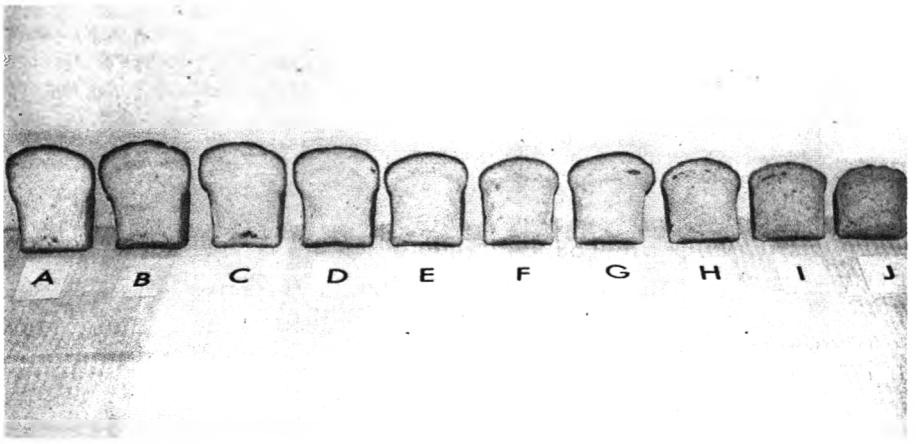

Fig. 2–Effect of BPC addition on bread loaf volume, color and texture. Protein contents are given in Table 6.

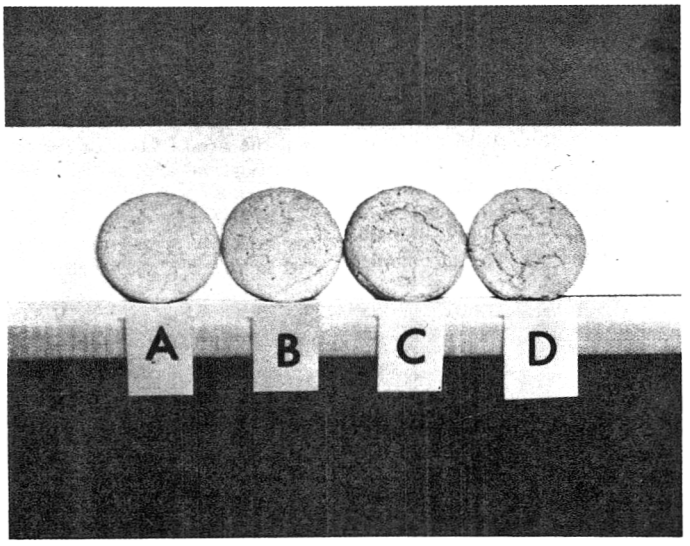

Fig. 3–Effect of BPC addition on the spread, texture and color of sugar cookies. Protein content of the samples is given in Table 7.

The effect of BPC on the baking qualities of cookies and bread was also tested. The effect of BPC fortification on the spread and height of a basic sugar cookie was measured using the procedure described by Fogg and Tinklin (1972) and Tsen et al. (1973). Bean protein fortification of white bread was evaluated using the procedure described by Marnett et al. (1973).

RESULTS & DISCUSSION

AUTOCLAVING the hammer milled bean flour prior to protein extraction reduced the amount of extractable protein by 30–40%. Therefore, any heat treatment to be used to destroy antinutritional factors in the bean would need to be performed on the extracted protein rather than the bean flour.

Table 1 shows the proximate composition of bean protein concentrate (BPC), the albumins, globulins and the residue and whey obtained from the bean extraction. The BPC fractions are composed of 65% globulins and 35% albumins. The globulin fraction, when isolated, contained the greatest protein content (91.04%). The isolated albumin fraction contained a lower protein content (75.98%), mainly because the large amount of carbohydrate (17.80%) in the albumin fraction. The dry residue and whey would have potential use as animal feeds because of their high carbohydrate and fiber content (83.33% in the residue) and the high proteinaceous material content (27.48% in dried whey).

Emulsion capacity tests, as summarized in Table 2, revealed that albumins have an exceptionally high oil binding capacity, though their stability was poor. The albumins have a greater emulsion capacity compared to the globulins. The low emulsion capacity of the globulins is reflected in a low emulsion capacity of the BPC. The globulins showed good stability in the heated sausages used in the emulsion stability test. Even though the albumins had a high emulsion capacity, this strength was not present in the emulsion stability of the albumins. Table 3 gives the emulsion stability of the various protein powders.

Whipping properties were determined by using whipping times that demonstrated the best foaming ability for the various proteins tested. These were 4 min of high speed whip for egg, 15 min for albumins, 9 min for globulins and 12 min for the globulin and albumin mixture. The foaming ability of the globulins increased with concentration, as did that of the albumins. At a protein level of 3–5%, the proteins demonstrated the highest amount of syneresis or foam instability. These results are presented in Table 4.

The foaming abilities were optimal at: pH 5.0–6.0 for globulins, pH 7.0 for albumins and pH 7.0 for BPC. Greatest foam stability was at a pH of 4.0 for all these products, even though this was not the optimal pH for foaming ability of the albumins and BPC. Table 5 gives data describing the effect of pH on the foaming characteristics of the bean proteins.

Preheating the protein dispersions at various temperatures demonstrated that heating increases foaming ability. The albumins had an optimal foaming ability and foam stability after heating to 70°C, while the globulins demonstrated greater stability at 85°C.

Washing globulins and albumins with acetone and ethyl alcohol to remove any residual lipids present did not increase their foaming ability or stability.

Table 6 gives the data which describe the effect of BPC addition on the loaf volume and protein content of white bread. Figure 2 shows the actual loaves of bread described in Table 6. The addition of the bean protein concentrate causes a reduction of the loaf volume. Above 18% protein, the bread was too dense to have an acceptable texture to the crumb. BPC does not give the bread any undesirable color, flavor or aroma when incorporated at levels shown in Table 6.

The addition of BPC to a sugar cookie recipe at levels replacing 10, 20 and 30% of the soft wheat flour enhanced the width:height ratio, or spread, of the cookies during baking. Again, no off color, aroma, flavor or unusual texture were imparted by the addition of BPC up to levels replacing 30% of the flour. Figure 3 shows the actual cookies described in Table 7.

Table 1—Proximate analysis of fractions from the wet fraction of the Great Northern bean

Fraction	Yield (% of whole)	% Protein	% Fat	% Ash	% Moisture	% Carbohydrate[b]
Milled bean	100	20.76	3.12	4.36	10.23	61.53
BPC[a]	16.43	84.38	0.97	1.60	2.85	10.20
Globulins	10.17	91.04	1.29	1.08	3.28	3.31
Albumins	6.26	75.98	0.87	3.66	1.69	17.80
Whey (dry)	8.14	27.48	1.13	9.43	10.67	51.29
Residue (dry)	75.43	7.41	2.37	3.41	3.48	83.33

[a] Bean Protein Concentrate contains both the globulins and albumins in a ratio of 1.62:1:00.
[b] Carbohydrate content was determined by difference.

Table 2—Emulsion capacity of various protein fractions from the Great Northern bean[a]

Protein	Emulsion capacity: ml oil emulsified / 100 mg protein	Emulsion capacity: ml oil emulsified / gram of sample
Nonfat dry milk	28.8	93
Globulins	12.4	113
Albumins	26.8	204
BPC	15.4	131

[a] All values represent means of triplicate analyses.

Table 3—Emulsion stability of the various protein fractions obtained from the Great Northern bean[a]

Fraction	Emulsion stability (ml released/10g of emulsified meat): Fat	Emulsion stability (ml released/10g of emulsified meat): Water + Suspension solids
Control[b]	0.09	0.5
Globulins	0.03	0.8
Albumins	0.04	1.3
BPC	0.13	1.2

[a] All values represent means of 10 sausages.
[b] Control contains added nonfat dry milk.

Table 4—The foaming and foam stability of bean proteins[a]

Protein	Protein content (%)	Vol increase[b] (%)	Syneresis (%)
BPC	1	195	45
	3	215	62
	5	220	67
	10	175	45
Albumins	1	325	22
	3	325	46
	5	386	46
	10	382	37
Globulins	1	168	40
	3	162	55
	5	178	55
	10	185	46

[a] All values are means from triplicate analyses.
[b] All whipping was done at pH 4.

Table 5—The effect of pH on the foaming and foam stability of bean protein[a]

Protein	pH	Vol increase (%)	Syneresis (%)
BPC	4	208	45
	5	202	50
	6	200	55
	7	225	66
	8	180	45
Albumins	4	328	36
	5	478	68
	6	504	76
	7	589	68
	8	425	53
Globulins	4	168	40
	5	208	44
	6	200	44
	7	170	47
	8	125	40

[a] All values are means of triplicate analyses.

Table 6—The effect of BPC addition on the loaf volume and protein content of white enriched flour bread[a]

Loaf	Volume (ml)	BPC added (g)	Protein content (%)
A	191.5	None	7.61
B	170.5	1.23	12.76
C	169.0	1.85	14.22
D	138.5	2.46	15.06
E	136.0	3.08	16.64
F	122.0	3.69	16.97
G	117.5	4.30	17.60
H	109.0	4.92	17.36
I	107.5	5.54	17.23
J	100.0	6.15	18.40

[a] All values are means of duplicate analyses.

Table 7—The effect of BPC addition on the spread and protein content of sugar cookies[a]

Cookie	BPC added (g)	Width:Height ratio	Protein content (%)
A	None	6.80	6.24
B	5.63	7.37	9.60
C	11.25	7.63	13.49
D	16.88	8.33	14.75

[a] All values are means obtained from six individual cookies.

CONCLUSION

THE EXTRACTION of Great Northern beans (*Phaseolus vulgaris*) with 2% NaCl yields a high protein fraction (Bean Protein Concent ate, or BPC) which contains 65% globulins and 35% albumins. The emulsifying characteristics of the bean proteins are classified as fair. The albumins have a good emulsifying capacity but have a poor emulsion stability, whereas the globulins have poor emulsion capacity but fair emulsion stability.

The bean proteins do foam when whipped, with the albumin fraction demonstrating good foaming capacity and stability at a protein level of 5% and at pH 4–5. The globulins have less foaming ability than do the albumins but do have slightly better foam stability.

The addition of BPC to white bread caused a drop in loaf volume as the amount of added BPC and protein content of the bread increased. The bean proteins when added to sugar cookies enhanced the width:height ratio.

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BIOCHEMICAL PROPERTIES OF ALFALFA PROTEIN CONCENTRATE

INTRODUCTION

PHOTOSYNTHESIS produces enormous amounts of food in the form of leafy green plants. This source of protein has remained unexploited in the diet of man, due partly to the low protein concentration in the leaves and the presence of fibrous material which can hinder digestion. Recent technological advances, however, have made it possible to separate the protein in a leaf from the accompanying fibrous materials making the protein more usable by man.

Several procedures have been recommended to prepare leaf protein concentrate (LPC) (Chayen et al., 1961; Morrison and Pirie, 1961; Huang et al., 1971; Lazar et al., 1971). A combination of pulping and pressing is generally recommended to extract juice from the leaves. Following its separation from the fibrous material, the juice can be filtered or centrifuged to remove suspended solids. Centrifugation will separate cytoplasmic from chloroplastic protein (Festenstein, 1961; De Fremery et al., 1973). Following centrifugation, the juice is an opaque brown color.

Protein in either the green or brown plant express juice can be coagulated by aging, adjustment of pH to 3–4, application of heat, a combination of heat and acid, or by the use of organic solvents (Chayen et al., 1961; Morrison and Pirie, 1961; Huang et al., 1971; Lazar et al., 1971; De Fremery et al., 1973).

Several factors have been observed which can influence the extractability of protein from leaves. These include the plant species and the stage of maturity (Lugg and Weller, 1944; Lexander et al., 1970), pH (Lugg, 1939; Festenstein, 1961; Betschart and Kinsella, 1973), the presence of phenolic compounds and oxidative enzymes (Loomis and Battaile, 1966), and proteolytic activity at temperatures greater than 30°C (Singh, 1962; De Fremery et al., 1972).

Acceptability of a protein concentrate in the diet will determine its future role in protein supplementation. Successful incorporation of a protein into a foodstuff is dependent on its functional properties, which can be influenced by the composition of proteins found in the concentrates. One portion of this study is devoted to the characterization of proteins in alfalfa protein concentrate (APC). The second portion deals with the identification of a plant component responsible for a color problem observed by the authors in the production of APC. At an acidic pH, APC is a light tan color; but when the pH is adjusted above pH 6.0, the protein concentrate turns a bright yellow-green. A similar problem was observed in protein concentrates prepared from sunflower seeds (Carter et al., 1970).

MATERIALS & METHODS

ALFALFA used in this investigation was the Dawson variety harvested at 1/10th bloom, immediately placed on dry ice, and transported to the laboratory where it was stored at −20°C. As needed, samples of frozen alfalfa were chopped in a Hobart Silent Cutter and processed to yield centrifuged alfalfa juice, whey and alfalfa protein concentrate (APC) using the procedure outlined in Figure 1.

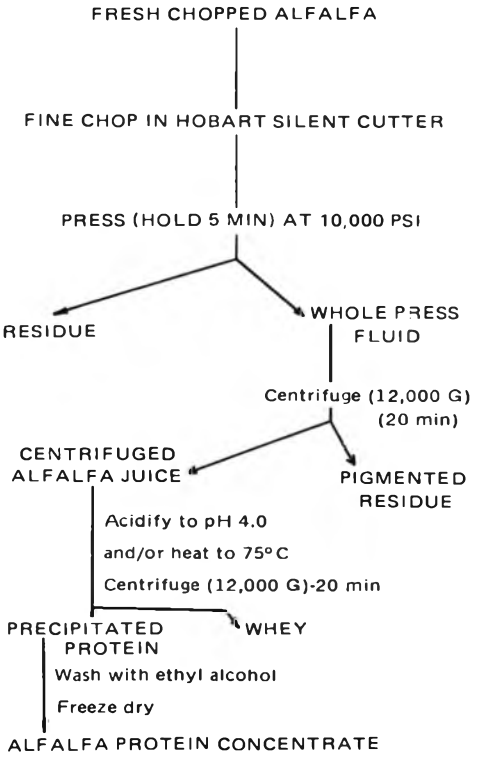

Fig. 1—Flow diagram showing methods used to obtain centrifuged alfalfa juice, whey and APC from whole alfalfa.

Sephadex G-50 fractionation of centrifuged alfalfa juice and whey

A 2 × 90 cm Sephadex G-50 column was used to fractionate the centrifuged alfalfa juice and the whey over a period of 96 hr at 4°C. A pH 5.8, 0.1M phosphate-1M NaCl buffer was used to fractionate a 3.5 ml concentrated sample. A 50% concentration of the samples was achieved by mixing 2.0g dry Sephadex G-10 with 11.0 ml of sample and centrifuging (1,500 × G for 20 min). Each fraction was analyzed for protein using Folin-Ciocalteau reagent. An ultraviolet absorption spectrum was determined for each fraction.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was used to separate the protein components of centrifuged alfalfa juice. Gels were stained for 30 min in Amido Swartz 10B stain (1.0% dye in a 5:5:1 solution methanol, distilled water and acetic acid) and subsequently destained for 24 hr in a 5:5:1 solution of methanol, distilled water and acetic acid.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Molecular weights were estimated using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis according to the method of Weber and Osborne (1969). The gels were stained for 6 hr in Coomasie Blue Stain (Bennet and Scott, 1971) and destained for 24 hr in 7% acetic acid. Gels were scanned to determine relative migrations using a Densicord Densitometer.

Isoelectric focusing

The protein fraction eluted from the Sephadex G-50 column was isoelectrically focused using a LKB 8101 Ampholine Electro Focusing Apparatus. All focusing runs were conducted for 48 hr at 4°C at a constant voltage of 800 volts. Isoelectric points were determined using a Corning Model 10 pH meter.

Isolation and identification of chlorogenic acid

Chlorogenic acid was isolated from the Sephadex G-50 fractions using the procedure of Milec et al. (1968). Identification of the isolate was made by comparing its infrared and ultraviolet absorption spectra to those of a pure sample of chlorogenic acid. The infrared spectrum was prepared using a Beckman IR-5A Infrared Spectrophotometer. A Hitachi Perkin-Elmer 139 UV-VIS Spectrophotometer was used to obtain the ultraviolet absorption spectrum.

Reducing agents and the recovery of protein from alfalfa juice

The effect of sodium sulfite and ascorbic

acid on the recovery of protein from fresh alfalfa plants was determined using 100g alfalfa samples to which 25 ml of either distilled water or various concentrations of the reducing agents, adjusted to pH 5.8, had been added prior to chopping in the Hobart Silent Cutter. The protein contained in the APC prepared from such samples was quantitated using Biuret reagent. The protein in the centrifuged alfalfa juice was analyzed using the UDY dye binding technique.

Decolorization of centrifuged alfalfa juice

Centrifuged alfalfa juice was decolorized by filtering it through a bed of activated charcoal (3:1, juice:charcoal, v:w) by suction filtration. Polyacrylamide gel electrophoresis, as previously described, was used to determine if the protein pattern of the decolorized alfalfa juice was the same as that observed from the centrifuged alfalfa juice.

Sephadex G-15 fractionation of centrifuged alfalfa juice

A 1 × 30 cm column of Sephadex G-15 was used to compare the composition of decolorized alfalfa juice and its whey to centrifuged alfalfa juice and its whey. A pH 5.8, 0.01M phosphate buffer was utilized to fractionate 0.5 ml samples over a period of 12 hr using a flow rate of 0.2 ml per minute. Folin-Ciocalteau reagent was used to analyze the protein in each fraction, and an ultraviolet absorption spectrum of each fraction was prepared. Folin-Ciocalteau reagent is specific for tyrosine, either in its free form or when in the protein structure. Therefore caution should be used when using this reagent on crude protein preparations.

Enzyme assay

The rate of hydrolysis of APC formed by heat-acid precipitation of centrifuged alfalfa press juice and decolorized alfalfa juice was compared using both trypsin and chymotrypsin digestion. A 1.0 ml sample of enzyme containing 1.0 mg protein per ml was added to a 5.0 ml suspension of protein with constant stirring at 37°C. Both solutions had previously been adjusted to pH 9.5. Change in the pH of the solution over time was monitored with a Corning Model 10 pH meter to determine the rate of hydrolysis.

RESULTS & DISCUSSION

Sephadex G-50 fractionation of centrifuged alfalfa juice and whey

Fractionation of centrifuged alfalfa juice (CAJ) using a Sephadex G-50 column yielded four fractions (Fig. 2). The first fraction was eluted with the void volume. Ultraviolet absorption spectra of the four fractions indicated that all four fractions had absorbance bands characteristic of proteins. Fractions 3 and 4 were yellow in color and had an additional absorbance band at 325 nm. Folin-Ciocalteau reagent indicated that fractions 1, 2 and 3 were protein positive (Table 1).

The elution pattern of centrifuged alfalfa juice containing 2% added sodium sulfite was identical to that of the centrifuged alfalfa press juice to which no sodium sulfite had been added during chopping. The ultraviolet absorption spectra of all four of the components from the sodium sulfite treated juice were also identical to those seen in untreated centrifuged alfalfa juice. The presence of protein was verified in fractions 1 and 2 of the centrifuged alfalfa juice with sodium sulfite. A total of 113.6 mg of protein was found in the fractions from centrifuged alfalfa juice which contained no added sodium sulfite, whereas fractions from the centrifuged juice with sodium sulfite added contained 298.8 mg of protein.

A sample of centrifuged alfalfa juice which had been dialyzed against water was separated by Sephadex G-50 chromatography into three fractions. The ultraviolet absorption spectra of fractions 1, 2 and 3 showed maximum absorption at 275 nm. Fractions 1 and 2 had an additional absorbance band at 325 nm. Folin-Ciocalteau reagent indicated the presence of 10.6 mg of protein in fraction 1 and 6.6 mg of protein in fraction 2. This is 76% and 2.5% of the protein that was found in fractions 1 and 2, respectively, of the nondialyzed sample of alfalfa press juice.

Sephadex G-50 elution patterns of whey both from alfalfa juice which had undergone heat-acid precipitation and from alfalfa juice which had undergone only heat precipitation contained three fractions (Fig. 3). No 280 nm absorbing material was observed in the void volume of either fraction. Similar ultraviolet absorbance spectra were observed for the three fractions, with absorption maxima at 270 nm. Fractions 3 and 4 had an additional absorbance band at 320 nm.

The entire amount of protein precipitated during the heat-acid treatment of centrifuged alfalfa juice was primarily in

Table 1—Analysis of protein in Sephadex G-50 fractions using Folin-Ciocalteau reagent

	mg Protein observed in Sephadex G-50 fractions			
	Fraction number			
Sample	1	2	3	4
Alfalfa juice	13.5	93.6	6.5	*
Alfalfa juice with Na sulfite	36.9	261.9	*	*
Dialyzed alfalfa juice	10.6	6.6	*	*
Heat-acid whey	—	74.7	*	*
Heat only whey	2.25	77.0	5.6	*

* Protein negative with Folin-Ciocalteau reagent

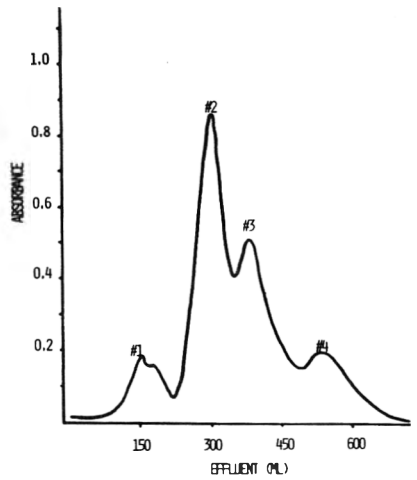

Fig. 2—Elution pattern obtained from fractionation of centrifuged alfalfa juice using Sephadex G-50 gel filtration chromatography.

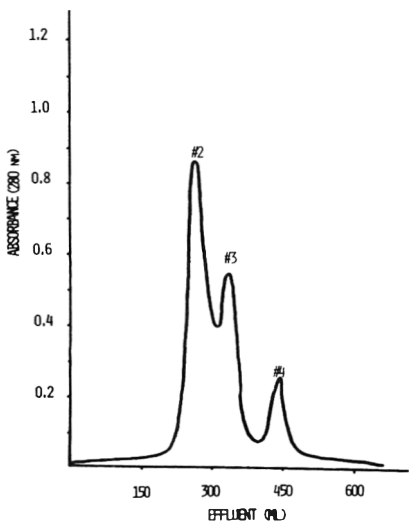

Fig. 3—Elution pattern obtained from fractionation of whey from either heat-acid or heat only precipitation of alfalfa juice using Sephadex G-50 gel filtration chromatography.

the first two fractions eluted. Whey from juice precipitated with heat alone contained some protein in fraction 2 and a large amount in fraction 3. A trace of protein was found in the void volume of the treated sample with heat only. A total of 85 mg of protein was found in this sample (Table 1).

Several observations can be drawn from the fractionation of centrifuged alfalfa juice and whey using Sephadex G-50. First, it appears that all of the protein in the first fraction precipitates upon the addition of heat and acid to centrifuged alfalfa juice. This conclusion can be drawn from the absence of this component in the fractionated whey samples. The majority of the proteinaceous material observed in the other fractions remains after heat-acid precipitation as indicated by Folin-Ciocalteau reagent. The protein which precipitates (APC) upon the addition of heat and acid to the brown centrifuge juice is largely water soluble in nature, since it does not precipitate when the brown juice is dialyzed against distilled water. Second, a large portion of the proteinaceous material present in centrifuged alfalfa juice is in the form of free amino acids and peptides since it is lost upon dialysis in water. The second and third fractions from the centrifuged alfalfa juice contained 88.1% of the protein present. These proteins were not removed by heat-acid precipitation and were found to be dialyzable, which is characteristic of small molecular weight peptides and amino acids. This would imply that only 11.9% of the proteinaceous material in centrifuged alfalfa juice is theoretically recoverable as APC. Third, sodium sulfite improves the extractability of protein from alfalfa plants. Using Folin-Ciocalteau reagent, it was observed that alfalfa juice with sodium sulfite added contained 42.7 mg of protein per ml, whereas alfalfa juice with no sodium sulfite added contained only 16.2 mg of protein per ml of centrifuged alfalfa juice. The reducing agents may improve the solubility of the proteins present in the whole alfalfa plant (Fig. 4). The amount of APC produced was not proportionately as large, since some of the solubilized would not precipitate at pH 4.0 and 75°C. Reducing agents may also prevent the oxidation of chlorogenic acid to its quinone form, in which form it covalently binds to protein forming chromophores. It was observed that alfalfa protein concentrate formed from press juice to which sodium sulfite or ascorbic acid was added was lighter in color.

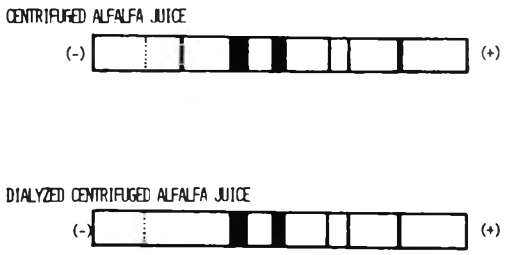

Fig. 5—Polyacrylamide gel electrophoresis of centrifuged alfalfa juice and dialyzed centrifuged alfalfa juice.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis of alfalfa juice supported the theory that the majority of the proteins in alfalfa are water soluble. Seven proteins were observed on the gel to which a sample of centrifuged alfalfa juice was added and six proteins were observed on the gel to which a sample of dialyzed alfalfa juice had been applied (Fig. 5).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Sodium dodecyl sulfate gel electrophoresis of alfalfa juice indicated that the proteins fell into two molecular weight ranges. The majority of the protein (93.7%) exhibited a molecular weight in the range of 25,000–60,000. The remaining protein (6.3%) was estimated to have a molecular weight of 600,000. Since sodium dodecyl sulfate (SDS) depolymerizes proteins, the large amount of

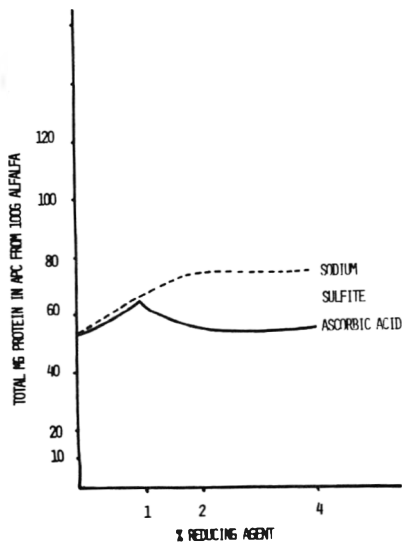

Fig. 4—The effect of reducing agents on the recovery of APC from centrifuged alfalfa juice.

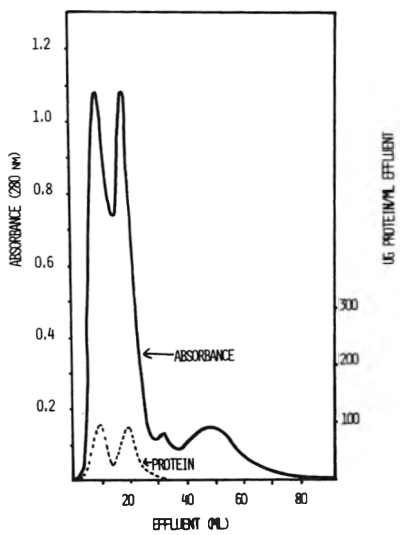

Fig. 6—Elution pattern of pigmented alfalfa juice fractionated using Sephadex G-15 gel filtration chromatography.

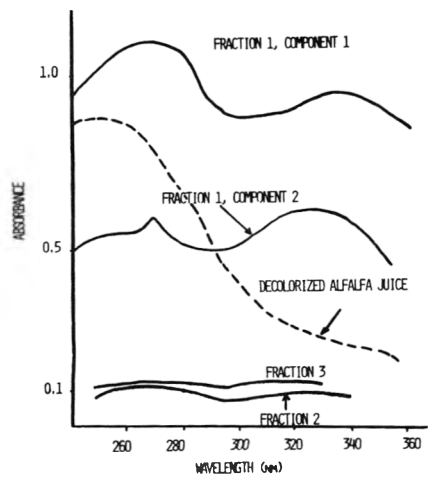

Fig. 7—Ultraviolet absorption spectra of fractions from Sephadex G-15 gel filtration chromatography of pigmented alfalfa juice and decolorized alfalfa juice.

Table 2–Isoelectric points of alfalfa protein components

Component	Isoelectric point
1	8.22
2	6.40
3	5.50
4	4.49
5	3.69

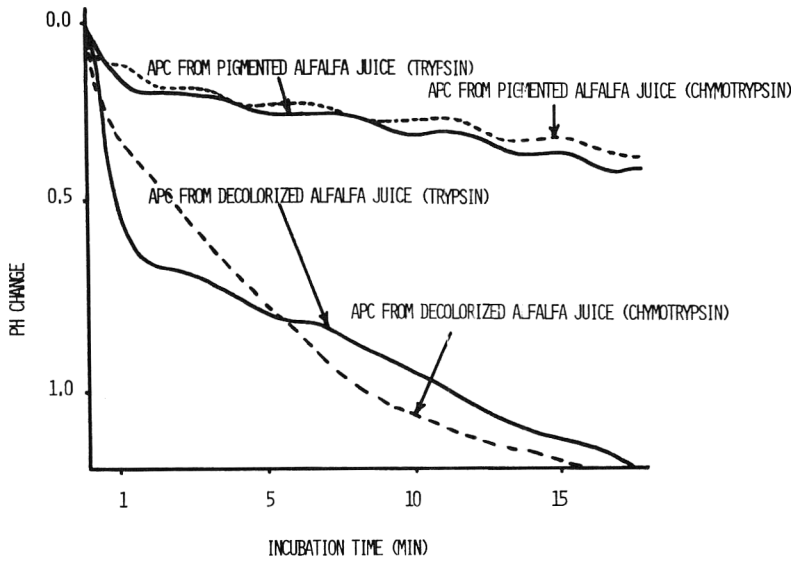

Fig. 8–Enzyme hydrolysis of APC from decolorized alfalfa juice and pigmented alfalfa juice using either trypsin or chymotrypsin.

small molecular weight protein may well be an artifact of SDS treatment.

Isolectric focusing

Five components were observed when protein from centrifuged alfalfa juice was isoelectrically focused (Table 2).

Isolation and identification of chlorogenic acid

Isolation of chlorogenic acid from centrifuged alfalfa juice yielded a bright yellow powder. Spectra from infrared analysis of pure chlorogenic acid and the isolate sample had absorbance bands of equal intensity at 2,850, 1,450 and 1,370 cm^{-1}. The ultraviolet absorbance spectra for the pure chlorogenic acid and the isolated sample were identical. A broad band from 320–330 nm with a maximum at 324 nm was observed.

Decolorization of centrifuged alfalfa juice

The brown centrifuged alfalfa press juice was colorless after it had been filtered through activated charcoal. Polyacrylamide gel electrophoresis demonstrated that the protein composition of the decolorized juice was similar to the pigmented alfalfa juice. A white flocculent precipitate (APC) appeared when heat (75°C) and acid (pH 4.0) were applied to the decolorized solution.

Sephadex G-15 fractionation of centrifuged alfalfa juice

Fractionation of centrifuged alfalfa juice with Sephadex G-15 yielded three fractions (Fig. 6). Fraction 1 was found to contain two protein components when tested with Folin-Ciocalteau reagent. The ultraviolet absorbance spectra of both components indicated absorbance bands at 270 and 325 nm.

Fractions 2 and 3 exhibited maximum absorbance at 270 and 325 nm. These fractions were protein negative when analyzed with the Folin-Ciocalteau reagent.

The whey formed from a heat-acid precipitation of proteins in pigmented centrifuged alfalfa juice had an elution pattern similar to that observed for the pigmented centrifuged alfalfa juice except that the first fraction was resolved into two components. Folin-Ciocalteau reagent indicated that the two components contained protein but to a lesser degree than that observed in the centrifuged alfalfa juice itself.

A difference was noted in the ultraviolet absorbance spectra of the two protein components. An absorbance band at 324 nm was not present in Component 1 but could be observed in Component 2 (Fig. 7). Each component had an absorbance maximum at 270 nm. These results suggested that the protein was bound to chlorogenic acid in the alfalfa juice, and that they co-precipitate upon treatment with heat and acid. This is particularly true in the case of the higher molecular weight component (Component 1).

One component was eluted when decolorized alfalfa juice was fractionated with Sephadex G-15. Protein is present in this fraction as was indicated by the Folin-Ciocalteau reagent. The ultraviolet absorption spectra of this faction contained one absorbance band at 270 nm.

The elution pattern of whey from a decolorized heat-acid precipitated alfalfa juice was identical to that of the untreated decolorized alfalfa juice. The ultraviolet absorbance spectra were also the same.

Decolorization of alfalfa juice using activated charcoal is a potential method for improving the quality of APC, due to its ability to decrease the chlorogenic acid content. A drawback was observed using this technique to recover white APC from the juice. After it had been decolorized, the alfalfa juice contained only 1/3 of its original protein. This was likely due to the retention of proteins which were bound to chlorogenic acid or other pigments on the activated charcoal. Further work in this area is warranted.

Enzyme assay

The rate of hydrolysis by both trypsin and chymotrypsin is greater for APC produced from decolorized alfalfa juice than for APC produced from pigmented alfalfa juice (Fig. 8). It is possible that the binding of chlorogenic acid to the protein in APC affects the binding of these enzymes influencing the digestibility of the protein concentrate.

SUMMARY & CONCLUSIONS

SEVEN PROTEIN FRACTIONS were observed to be present in alfalfa juice with the majority of those proteins demonstrated to be water soluble in nature. Five isoelectric focusing components were observed, four of which were in the acidic pH range.

Chlorogenic acid was isolated and identified in alfalfa juice. A solution of pure chlorogenic acid and distilled water is clear at an acidic pH but turns bright yellow upon the addition of 1N NaOH. The Sephadex G-15 gel filtration chromatography of centrifuged alfalfa juice and decolorized alfalfa juice and the enzyme hydrolysis experiment suggest that chlorogenic acid binds to the proteins in APC. The binding of chlorogenic acid to the proteins in APC appears to be the cause of the conversion of tan APC to yellow-green APC at a pH greater than 6.0.

Decolorization of alfalfa juice by filtering it through activated charcoal is suggested as a means of reducing the amount

of chlorogenic acid present in centrifuged alfalfa juice. The addition of sodium sulfite or ascorbic acid to alfalfa during chopping is shown to improve both the quality and quantity of alfalfa protein concentrate recovered.

Heat and acid precipitation was shown to be more efficient than precipitation by heat alone in the recovery of APC from centrifuged alfalfa juice.

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TRITICALE, SOY-TVP AND MILLET BASED DIETS AS PROTEIN SUPPLIERS FOR HUMAN ADULTS

INTRODUCTION

BECAUSE of expanding world human populations and inequities in distribution of resources among these people, the already heavy dependence of human beings upon cereal/plant resources of protein is almost certain to increase. Millet grain, triticale grain and soybean are representative of rather different sources of dietary protein for the human. These sources are examples of the very old, the very new, the traditional and the nontraditional.

While millets are not commonly consumed for human food in the United States, they do constitute a recognized part of human diets in several parts of the world, particularly India. Several studies involving use of human subjects in the study of protein value of millets have been reported (Ambegaokar et al., 1965; Daniel et al., 1965a, b). Research on protein value of triticale flour and soy meat-analogs for humans has been recently reviewed by Kies and Fox (1973, 1974).

Unlike laboratory test diets, self-selected diets of humans usually contain mixtures of foods; hence, mixtures of protein food resources. These have frequently been found to have protein value superior to any of the constituent sources when fed alone. This effect does not always occur and is not always predictable. A favorable effect is usually credited as being due to an increased supply of the first limiting amino acid. However, desirability of amino acid proportionality patterns is not completely determined by supply of the first limiting amino acid. Furthermore, amino acid proportionality patterns are not the sole determinants of protein quality.

The objectives of the four studies composing the present project are as follows:

(1)To define the relative protein value of a soy-TVP product (a meat-analog produced from extruded defatted soybean meal), a triticale flour, and a millet flour for human adults;

(2)To determine the effect on protein value for humans of adding triticale to millet flours;

(3)To determine whether the increase in lysine content or the increase in nonspecific nitrogen intake (total protein intake) was the primary cause of the increased protein value resulting from addition of triticale flour to millet flour; and

(4)To compare the effect on protein nutriture of humans of additions of millet flour or triticale flour to a soy-TVP® diet.

PROCEDURE

EXPERIMENTAL PLANS for the four studies of the project are given in Tables 1–4. Each study was 18 days in length and was divided into a 2-day nitrogen depletion period, a 4-day nitrogen adjustment period, and three experimental periods of 4 days each. Order of the experimental periods in each study was randomly arranged for each of the six to seven individuals who were subjects during each trial.

Nitrogen intake during the nitrogen depletion period was 0.8g per subject per day as provided by the ordinary foods in the basal diet (Table 1). This procedure has been found in earlier studies in this laboratory to hasten the adjustment of subjects to low protein diets.

During the adjustment period nitrogen intake was maintained at 4.8g per subject per day: 4.0g nitrogen from reconstituted dry skim milk and 0.8g nitrogen from the basal diet. Previous research from this laboratory indicates this to be a good level of total nitrogen intake for testing protein value of food products in that it is less than optimal for even those products with ideally balanced amino acid patterns. Protein quality testing cannot be done at optimal levels of protein intake in that differences in protein quality cannot be defined. However, some time must be allowed for adjustment to this low level of total nitrogen intake. This was the primary purpose of this period.

Diets fed during the experimental periods of each study are defined in Tables 1–4. These were designed to meet objectives of the four studies. Energy intake for each subject was maintained constant at that level necessary for maintenance of body weight by adjusting intake of extra energy foods–starch bread, butter oil, carbonated beverages and hard candy. Vitamin and mineral supplements were included as defined in Table 1. Specific methods used in preparation of food items including the triticale or millet breads and soy-TVP casseroles have been defined in earlier studies (Kies and Fox, 1970, 1971).

Table 1–Comparative protein value of soy-TVP, millet flour and triticale flour for humans

Period[a]		No. of days	Test protein source[b] Type	Intake g source/day	Intake g N/day	Total N[c] intake g N/day	N excretion[d] Urine g N/day	Feces g N/day	N[d] balance g N/day
Depletion		2	–	–	–	0.80	–	–	–
Adjustment		4	Milk	79.70	4.0	4.80	–	–	–
Random Order	Exp. 1	4	Soy-TVP	52.98	4.0	4.80	4.18	1.04	−0.42e
	Exp. 2	4	Triticale	156.07	4.0	4.80	4.24	1.04	−0.58e
	Exp. 3	4	Millet	213.68	4.0	4.80	3.99	1.80	−0.99f

[a] Order of experimental periods 1–3 randomized for each of the experimental subjects.

[b] Milk was a dry, skim milk product. TVP® (textured vegetable protein) is an extruded defatted soy-meal product processed to resemble ground beef (Archer Daniels Midland Co., Decatur, IL 62525). The triticale flour was a 100% whole grain flour (Triticale Foods Corp., Muleshoe, Texas). The proso millet flour was a 100% whole grain flour (Panicum miliaceum, a white seeded type referred to as common white, not a variety).

[c] Includes 0.80g N/day provided by basal diet. The basal diet consisted of applesauce (100g), peaches (100g), tomato juice (100g), green beans (100g), starch bread, jelly butter oil, tea or coffee (7g), carbonated beverages, bouillon (3.5g). A vitamin supplement provided 2,000 IU vitamin A, 900 IU vitamin D, 2 mg thiamin, 50 mg ascorbic acid, 1 mg vitamin B_6, 2.5 mg riboflavin, 1 mg pyridoxine, 1 μg vitamin B_{12}, 20 mg niacinamine, 1 mg Ca pantothenate A mineral supplement provided the following (g/subject/day): Ca, 1.00; P, 1.00; Mg, 0.239; Fe, 0.0.8; Cu, 0.0024; I, 0.00018; Mn, 0.0024; Zn, 0.00108. NaCl was allowed ad libitum. The ordinary foods, including yeast were relied upon to give additional amounts of these and other vitamins and minerals such as biotin, folic acid, and vitamin E.

[d] Each figure is the mean values of all subjects over a 4-day period. Different letters following the mean denote values differing from one another at greater than 1% level of significance.

The 26 young men and women (mean age 21 yr) who were subjects for the study were all students of the University of Nebraska and all (except one) were either seniors or graduate students in the area of foods and nutrition. All maintained their usual working, living and studying routines except for eating of the experimental diet in the metabolism laboratory of the Dept. of Food & Nutrition and for making collections of excreta. All were volunteers who were approved for participation by the Student Health Div. of the University of Nebraska.

The nitrogen balance technique was used as the criterion of evaluation. Urine, feces and food were collected and prepared for analyses as described previously (Linkswiler et al., 1958) were analyzed for nitrogen content by the boric acid modification of the Kjeldahl method (Scales and Harrison, 1920). Urine samples were preserved under toluene and analyzed daily for 24-hr excretion of nitrogen and creatinine (Folin, 1914). Daily fecal nitrogen values were based on 4-day composites.

Amino acid composition of the three test products was determined by the Dept. of Agronomy, University of Nebraska, via auto analyzer methodology.

Statistical analyses of data, including analyses of variance and Duncan's Multiple Range Test, were by the Statistical Laboratory of the

Table 2—Effect of partial replacement of millet flour with tritical flour on N balances of humans

	Period[a]	No. of days	Millet intake[b] Flour g/day	Millet intake[b] N g/day	Tritical intake[b] Flour g/day	Tritical intake[b] N g/day	Total intake[c] Flour g/day	Total intake[c] N g/day	N excretion[d] Urine g/day	N excretion[d] Feces g/day	N^d balance g/day
	Depletion	2	–	–	–	–	–	0.8	–	–	–
	Adjustment	4	(4.0g N intake/day from milk)					4.8	–	–	–
Random order	Exp. 1	4	213.7	4.0	–	–	213.7	4.80	3.88	1.73	–0.81e
Random order	Exp. 2	4	160.3	3.0	53.4	1.4	213.7	4.80	3.95	1.61	–0.40f
Random order	Exp. 3	4	106.8	2.0	106.8	2.7	213.7	5.52	4.38	1.41	–0.27g

a,b,c,d See footnotes for Table 1.

Table 3—Comparative influence of added lysine or added nonspecific N on apparent protein value of millet for humans

	Period[a]	No. of days	N intake (g N/day) Millet[b]	Urea	Lysine	Total[c]	N excretion (g N/day)d Urine	Feces	N^d balance g N/day
	Depletion	2	–	–	–	0.80	–	–	–
	Adjustment	4	(4.0 N/day from milk)			4.80	–	–	–
Random order	Exp. 1	4	4.0	–	–	4.80	3.94	1.50	–0.64g
Random order	Exp. 2	4	4.0	0.72	–	5.52	4.32	1.41	–0.21e
Random order	Exp. 3	4	4.0	–	0.01	4.81	3.57	1.66	–0.42f

a,b,c,d See footnotes for Table 1.

Table 4—Comparative effect of triticale or millet flour addition to soy-TVP diet on protein value for human adults

	Period[a]	No. of days	Soy-TVP Source g	Soy-TVP N g	Triticale Source g	Triticale N g	Millet Source g	Millet N g	Total[c] N intake g N	N^d N excretion[d] Urine g N	N^d N excretion[d] Feces g N	N^d balance g N
	Depletion	2	–	–	–	–	–	–	0.80	–	–	–
	Adjustment	4	(4.0g N from milk)									
Random order	Exp. 1	4	52.98	4.0	–	–	–	–	4.80	4.19	0.99	–0.38e
Random order	Exp. 2	4	26.49	2.0	106.84	2.72	–	–	5.52	4.13	1.09	+0.30f
Random order	Exp. 3	4	26.49	2.0	–	–	106.84	2.00	4.80	3.53	1.57	–0.30e

a,b,c,d See footnotes to Table 1.

Table 5—Amino acid patterns of test protein sources

Amino acid	Amount anico acid (g) per g of test protein N		
	Millet	Soy-TVP	Triticale
Lysine	0.095	0.393	0.186
Histidine	0.132	0.167	0.141
Arginine	0.177	0.469	0.291
Aspartic acid	0.377	0.745	0.378
Threonine	0.205	0.269	0.187
Serine	0.418	0.452	0.279
Glutamic acid	1.392	1.634	1.956
Proline	0.413	0.364	0.622
Glycine	0.138	0.263	0.249
Alanine	0.665	0.271	0.222
Half cystine[a]	(0.000)	(0.051)	(0.065)
Valine	0.310	0.325	0.263
Methionine	0.128	0.074	0.080
Isoleucine	0.269	0.306	0.220
Leucine	0.801	0.506	0.401
Tyrosine	0.114	0.201	0.119
Phenylalanine	0.339	0.343	0.266
Tryptophan[b]	(0.038)	(0.086)	(0.072)
N content (g N/g test product)	0.01872	0.07750	0.02563

[a] Cystine is subject to destruction by method used, hence, values listed are probably low.
[b] Estimated from handbook values.

Nebraska Agricultural Experiment Station, University of Nebraska.

RESULTS & DISCUSSION

AMINO ACID PATTERNS of the three test proteins are given in Table 5. Lysine and methionine are the two essential amino acids most frequently found to be first limiting in food products. Rank listing of the three test products in order of apparent suppliers of lysine is as follows: soy-TVP, triticale and millet. Soy-TVP on a 1g of test protein nitrogen basis apparently supplies nearly twice the amount of lysine as does triticale and nearly four times the amount as does millet. Millet, however, contains approximately 50% more methionine in comparison to both soy-TVP and triticale. Cystine can spare up to 90% of the methionine requirement of the human. Thus, the higher cystine contents of soy-TVP and triticale would tend to make differences in total sulfur-containing amino acid contents of the three test products less pronounced. Variety, growing conditions and processing are known to have an influence on total protein content and on amino acid proportionality patterns of triticale flour, soy-TVP and millet flour. Hence, values listed should not be assumed to be the same for all products.

Mean nitrogen balances of subjects as well as mean urine and fecal nitrogen excretion data from the four composite studies of the project are shown in Tables 1–4.

In the first study (Table 1), protein values of the soy-TVP and triticale flour were found to be superior to that of the millet flour on the basis of the nitrogen balance data. Although the nitrogen balance achieved while feeding soy-TVP was −0.42 vs −0.58 when the triticale flour was fed, these values were not significantly different. Fecal nitrogen excretion values (1.04, 1.14 and 1.80g per day for soy-TVP, triticale flour and millet flour, respectively) suggest that the poorer performance of the millet flour protein was at least in part due to its lower digestibility.

In the second study (Table 2) replacement of 25% and 50% of the millet flour with triticale flour resulted in a progressive improvement in nitrogen balance of subjects fed the mixtures. These mixtures were fed on the basis of equal quantities of flour rather than on the basis of equal quantities of nitrogen. Since the triticale flour had both higher protein and lysine, both lysine and nitrogen contents of the mixtures were increased as a result of progressively increasing the amount of triticale flour in the combination. The digestibility of the mixtures were also progressively improved as a result of this procedure. Other factors could also be involved in this apparent enhancement in protein value.

In the third study (Table 3) two of these factors were investigated. Nonspecific nitrogen in the form of urea or lysine supplements were added to the millet flour diets in the same levels as were added by substitution of 50% of the millet flour with triticale flour in the second study. Millet flour alone gave a nitrogen balance of −0.64g nitrogen/day. Addition of lysine gave a nitrogen balance of −0.42g, a statistically significant increase. However, urea supplementation gave a nitrogen balance of −0.21, an even greater increase. This would suggest that the improvement achieved by substitution of part of the millet flour with triticale flour was mainly due to the increase in total protein content of the mixture rather than to improvement in lysine content, although both factors were involved. The importance of total nitrogen intake or nonspecific nitrogen intake on apparent value of food products as sources of protein has been previously discussed (Kies, 1972; 1974).

In the fourth study, effect of substitution of part of a soy-TVP diet with millet flour or triticale flour on protein nutriture of human subjects was investigated (Table 4). When the triticale flour was substituted for the millet flour (equal quantity of flour basis) as a supplement to the soy-TVP diet, a significant increase in nitrogen retention occurred over that achieved when soy-TVP was fed alone or when the 50/50 soy N and millet N mixture was used. Addition of triticale resulted in an increase in both total protein (nitrogen) content and an improvement in the amino acid proportionality pattern. Although not tested, both factors were most likely involved. When 50% of the soy-TVP protein was substituted with millet protein, no significant change occurred in nitrogen balances of subjects although the quality of millet protein in the first study was found to be lower than that of the soy protein. Since lysine is the first limiting amino acid in millet protein and total sulfur-containing amino acids for soy, some mutual supplementary effects may be coming into play.

These results suggest that triticale additions may be advantageous in the case of relatively poor quality plant protein resources such as millet and also in the case of relatively good quality sources such as soy. Additional work is needed on the effects of mixing cereal/plant protein resources in human diets.

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INFLUENCE OF TEMPERATURE ON KINETIC PROPERTIES OF PHOSPHORYLASE FROM TWO VARIETIES OF POTATO TUBER

INTRODUCTION

STORAGE of potato tubers at low temperatures generally stimulates the conversion of starch to sugars (glucose, fructose and sucrose). The tendency of tubers to accumulate reducing sugars may be determined by variety (Akeley et al., 1965), cultural practices (Hart and Smith, 1966), physiological stage of development (Yamaguchi et al., 1966) and environmental parameters (Yamaguchi et al., 1964). The tendency of cold stored tubers to undergo "Maillard" browning during processing requires that the tubers be "conditioned" at higher temperatures which results in a reduced pool of reducing sugars and accordingly less "Maillard" browning during high temperature processing. Because of the economic advantages of processing tubers directly from cold storage, much attention has been given to understanding the mechanisms of control of carbohydrate metabolism in potato tubers.

Phosphorylase (α-1,4-glucan orthophosphate glucosyltransferase, E.C.2.4.1.1) is believed to play a major role in the metabolism of starch (Henderson, 1968; Nordin and Kirkwood, 1965; Whelan, 1961). Hyde and Morrison (1964) reported that phosphorylase activity was generally greater in tubers stored at 4°C than those conditioned for 4 wk at 21°C. Ioannou et al. (1973) reported only small differences in the phosphorylase content and minor differences in phosphorylase isozyme patterns of cold-stored and freshly harvested Kennebec tubers.

Ehmann and Hultin (1973) have shown that lactate dehydrogenase from chicken breast muscle becomes more efficient catalytically at low substrate concentrations as the temperature is lowered to 4°C. A decrease in Km with decreasing temperature has been demonstrated for muscle phosphorylase (Helmreich and Cori, 1964) and for several enzymes of poikilotherms (Hebb et al., 1972; Hochachka and Lewis, 1971).

The control of phosphorylase in muscle involves the interaction of adenosine-3′,5′-monophosphate (C-AMP) with the inactive phosphorylase kinase (Stadtman, 1970). At the present time, there is no evidence that the same system of phosphorylase regulation is operative in potato tubers. However, infiltration of potato tubers with C-AMP has shown some changes in reducing sugar content (Haard, 1973).

We have examined the effect of temperature on the kinetics of starch degradation by phosphorylase in extracts from two varieties of potato tubers: Kennebec, which accumulates reducing sugars during low-temperature storage, and Monona, which does not accumulate significant levels of reducing sugars during cold storage.

MATERIALS & METHODS

TUBERS (*Solanum tuberosum* var. "Kennebec" and "Monona") were grown on the Rutgers experimental farm under similar conditions. After harvest, the tubers were stored at 5°C for 6–12 wk. The tubers were taken out of cold storage, washed, peeled, frozen in liquid nitrogen, and powdered using an Omni Mixer (Servall). The powders were stored at −20°C until used.

Phosphorylase extracts were prepared by homogenizing 10g of powdered tissue with 10 ml of ice cold 0.5% sodium citrate–0.5% sodium dithionite in a Potter-Evelhjem-type homogenizer. The resulting homogenate was centrifuged at 0°C for 15 min at 15,000 × G. The supernatant fraction was decanted and dialyzed at 5°C overnight against 10 liters of 0.5% sodium dithionite, 10 mM sodium maleate, pH 6.3. The dialyzed extracts were centrifuged at 0°C for 10 min at 15,000 × G before assay.

Phosphorylase was assayed using a radiometric technique based on the assay of Gold et al. (1970). The reaction contained 10^6 CPM of $^{32}PO_4$ (ICN), 0.1% soluble starch (Fisher), 50 mM Tris (tris hydroxymethyl aminomethane), 50 mM sodium maleate, pH 6.3, and 20 mM NaH_2PO_4, pH 6.3. The reaction was started by the addition of 0.200 ml of potato extract to the reaction mixture. The temperature was controlled by placing the test tubes in circulating water baths (Forma Scientific, Blue "M") maintained at the desired temperature. Aliquots (0.200 ml) were taken from the reaction mixture initially and after the desired time (5–20 min) had elapsed. The aliquots were immediately placed into a tube containing 2 ml of 5% ammonium molybdate (containing 2.5% by volume of H_2SO_4) and 4 ml of isobutanol-benzene (1:1), and agitated for 15 sec using a Maximixer (Thermolyne). The organic phase was removed and the aqueous phase was again extracted with 4 ml of isobutanol-benzene to remove the remaining unesterified $^{32}PO_4$. After removing the organic phase, a 0.200 ml aliquot of the aqueous phase containing glucose-1-$^{32}PO_4$ was removed for scintillation counting using the cocktail described by Ziegler et al. (1957). The samples were counted using the full window of a Beckman Model LS scintillation counter.

The effect of temperature on the Km for phosphate was determined using phosphate concentrations of 2, 5, 8, 10, 12 and 15 mM at 5, 15, and 25°C and a reaction time of 5 min. The effect of 3′,5′-adenosine monophosphate (C-AMP) (Sigma) on the Kennebec extract was determined by adding 1 mM C-AMP and 1 mM theophylline (Sigma) to the reaction mixture.

RESULTS & DISCUSSION

THE Km for phosphate of the phosphorylase extracts from cold-stored Monona tubers (Fig. 1) was 9 mM and was not affected by changing the temperature from 25°C to 15°C or 5°C. Since there was no change in Km with changing temperature, the activity of the enzyme can be described by a linear Arrhenius relation. The Km for phosphate of the phosphorylase extracts of cold stored Kennebec tubers (Fig. 2) was 5.6 mM at 25°C, but increased to 10 mM at 15°C and 5°C. At low phosphate concentrations, the activity of phosphorylase in Kennebec tubers would be lower than would be predicted by the Arrhenius relation. Because Kennebec tubers accumulate reducing sugars when stored at low temperatures, it was anticipated that the phosphorylase activity might be higher than would be normally expected. The results indicate that phosphorylase may be even less active at low temperatures and that the degradation reaction catalyzed by phosphorylase is not a major factor in the accumulation of reducing sugars during cold storage of Kennebec tubers.

The change in Km with changing temperature cannot be readily explained. Ehmann and Hultin (1973) found a reduction of Km of lactate dehydrogenase with a reduction in temperature and attributed these changes to alterations of

the interaction between the subunits of the enzyme and changes in the water structure with changing temperatures. It is not possible to distinguish between changes in the water structure and changes in the interaction between the two subunits (Iwata and Fukui, 1973) of phosphorylase because of the limited data available.

The importance of C-AMP in the regulation of phosphorylase in animal systems has been widely studied (Stadtman, 1970), but has not been demonstrated in plant systems. The addition of 1 mM C-AMP and 1 mM theophylline to the assay

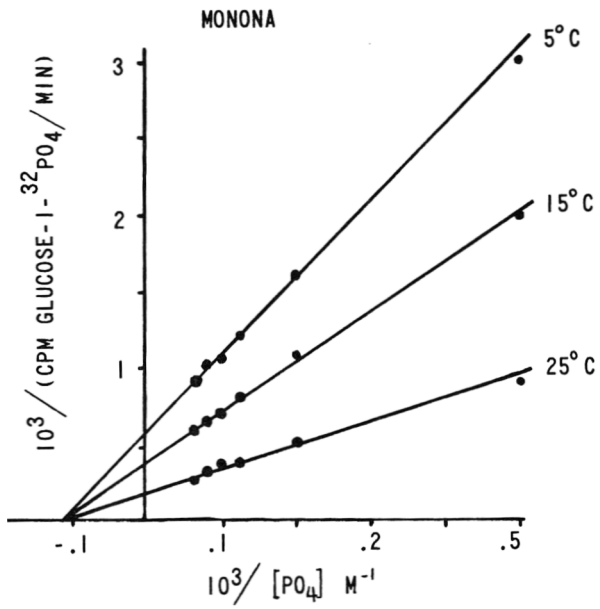

Fig. 1—Double reciprocal plot of phosphorylase extract from cold-stored "Monona" tubers. The phosphorylase activity was measured as the incorporation of $^{32}PO_4$ into glucose-1-$^{32}PO_4$ in a mixture containing 0.1% soluble starch, 50 mM Tris, 50 mM sodium maleate, pH 6.3, and NaH_2PO_4, pH 6.3, at the desired concentration. The assays were run at 5, 15 and 25° C. Data presented are for one experiment, but are representative of three experiments.

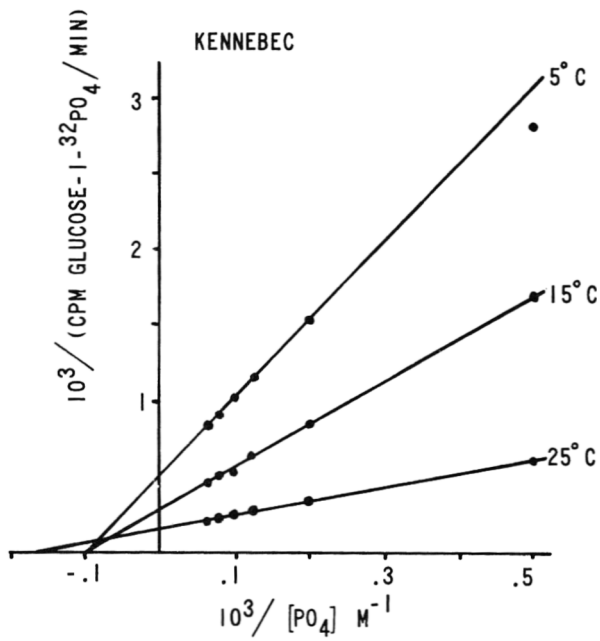

Fig. 2—Double reciprocal plot of phosphorylase extract from cold-stored "Kennebec" tubers. The phosphorylase activity was measured as the incorporation of $^{32}PO_4$ into glucose-1-$^{32}PO_4$ in a mixture containing 0.1% soluble starch, 50 mM Tris, 50 mM sodium maleate, pH 6.3, and NaH_2PO_4, pH 6.3, at the desired concentration. The assays were run at 5, 15, and 25° C. Data presented are for one experiment, but are representative of three experiments.

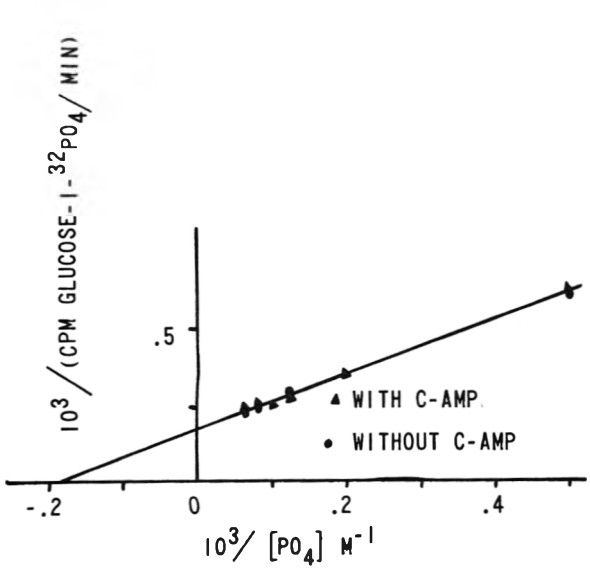

Fig. 3—Double reciprocal plot of phosphorylase extract from cold-stored "Kennebec" tubers. The phosphorylase activity was measured as the incorporation of $^{32}PO_4$ into glucose-1-$^{32}PO_4$ in a mixture containing 0.1% soluble starch, 50 mM Tris, 50 mM sodium maleate, pH 6.3, and NaH_2PO_4, pH 6.3, at the desired concentration (● ● ●) and with the addition of 1 mM C-AMP and 1 mM theophylline (▲ ▲ ▲). The assays were conducted at 25° C. Data presented are for one experiment, but are representative of three experiments.

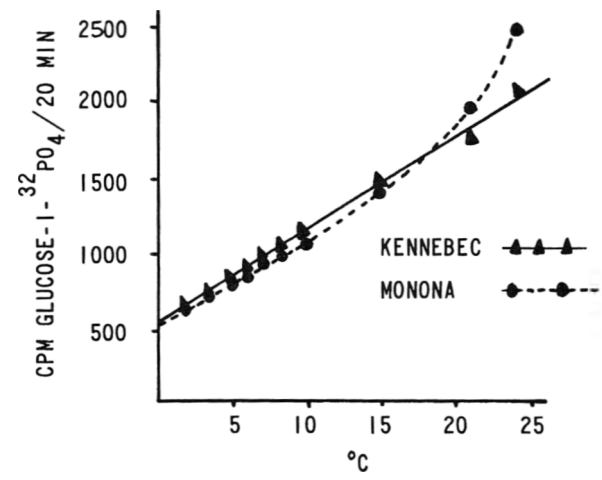

Fig. 4—The effect of temperature on maximum velocity for phosphorylase extracts from cold-stored Monona (▲--▲--) and Kennebec (●—●—●) tubers. Data presented are for one experiment, but are representative of three experiments.

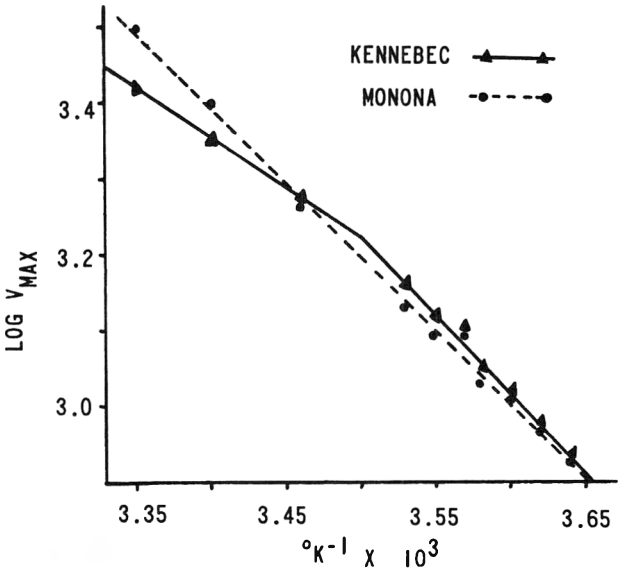

Fig. 5—Arrhenius plot of maximum velocity data for cold-stored Kennebec (●—●—) and Monona (▲— — —▲) tubers Data presented are for one experiment but are representative of three experiments.

had no effect on the kinetics of starch degradation when assayed at 25°C (Fig. 3). Similar results were obtained at 15°C and 5°C. It is possible that the C-AMP was hydrolyzed by a diesterase, but theophylline is an inhibitor of the diesterase in animal systems and may have prevented the hydrolysis. It is very possible that other labile components such as a phosphorylase kinase might be involved in a C-AMP mediated control system and, therefore, may not be active under the conditions of assay.

A plot of maximum velocity vs. temperature for phosphorylase extracts (Fig. 4) that were quantitatively prepared so that the volume of extract per gram of tissue was the same for both varieties shows some interesting differences. The maximum velocities obtained from Figure 1 and Figure 2 are somewhat different due to different concentrations of tissue in the extracts used in the Km study. The Monona extracts are more active at higher temperatures while the Kennebec extracts are relatively more active at the lower temperatures. That is, the temperature coefficient for phosphorylase from Monona is greater than that for Kennebec above 15°C. Plotting this data using an Arrhenius technique (Fig. 5) shows a break in the line obtained from Kennebec extracts near 12°C and the expected straight line for the Monona extracts. The change in the enzyme resulting in an increase in the activation energy at the lower temperatures could be caused by a temperature-induced conformational change.

The magnitude of the differences in phosphorylase activity of two extracts is not enough to account for the differences in the accumulation of reducing sugars during low temperature storage, but the Kennebec tubers do have slightly greater phosphorylase activity (Fig. 4) per gram of tissue than the Monona tubers when assayed at lower temperature.

The data presented do not explain the mechanism of reducing sugar accumulation during low-temperature storage of potato tubers and, for the most part, do not indicate that phosphorylase plays a regulatory role in this phenomenon. The regulatory components of the system may be labile and therefore may not have manifested themselves in our system. The control mechanism may be compartmentation of enzymes, substrates and inhibitors as suggested by Ohad et al. (1971) which would not have appeared in our study. It is also possible that other enzyme systems such as sucrose synthetase (Sowokinos, 1973) or invertase (Pressey, 1972) are the controlling factors. More investigation is needed to fully elucidate the mechanism of control of reducing sugar accumulation during low-temperature storage of potato tubers.

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APPARENT VISCOSITY AS A MEASURE OF MOIST MOUTHFEEL OF SWEET POTATOES

INTRODUCTION

THE CLASSIFICATION of sweet potatoes into moist and dry flesh is a very important quality factor in the industry. This property describes the mouthfeel characteristics after baking and is independent of water content (Nelson, 1973). The sweet potato (*Ipomoea batatas*) is one of the major vegetable crops grown in the United States. In the store, these vegetable crops are sold by a number of different names that give no indication of textural characteristics. Tests and descriptions are needed to provide a basic common terminology to distinguish moist and dry varieties so that buyers may have an idea of the type they are getting.

Several objective tests to determine texture have been employed in the past. Studies on firmness (Turnquist, 1948), penetration (Hilton and Evans, 1950) and tensile strength (Personius and Sharp, 1938) have been reported. Shama et al. (1973) have worked on the identification of stimuli controlling the sensory evaluation of viscosity considering the flow properties of foods. The appropriate rate of shear to determine the consistency of food behavior has been studied (Wood, 1968). It has been observed that in the process of chewing foodstuff, an isolated portion of the foodstuff is taken on the upper surface of the tongue and by raising and rolling the tongue around, a portion of the food is forced to flow between it and the roof of the mouth. The actual perception of stress or the rate of flow is registered by the sense organs (Scott Blair, 1966). Rheological properties of corn (Tung et al., 1974), including viscosity, have been studied to determine maturity. A pseudoplastic flow model with a yield stress was assumed and the flow behavior index found to be significantly correlated to the sweet corn maturity.

The objective of this research was to determine whether viscosity could be used as a means of classifying the sweet potato (varying variety and storage time) as to its kinesthetic (mouthfeel characteristics) quality.

EXPERIMENTAL

FOR THE PURPOSE of this research eight cultivars of sweet potatoes were chosen, viz. Pelican Processor, Australian Canner, Nugget, Jewel, Centennial, Porto Rico, Gem and an experimental cultivar designated as number 213X238-1 (referred throughout this manuscript as numbered). The sweet potatoes were U.S. Grade No. 1 obtained from the N.C. State University Agricultural Experiment Station. These sweet potatoes were harvested on Oct. 4, 1973 and some set aside for a test on uncured cultivars which was run on Oct. 10, 1973. The remaining were cured at 30°C and 80–90% relative humidity for 8 days and stored at 16°C and 55% relative humidity. The test dates for these roots were Oct. 17, Oct. 30, Nov. 14 and Dec. 12, 1973. These dates were chosen to detect changes of the properties with aging of the cultivars.

On each test date six or seven roots of each cultivar (roughly of same size) were baked in an electric oven at 190°C for 70 min. The time and temperature were determined on the basis of prior work (Nelson, 1973). Following baking, the roots were skinned and the flesh from the central part removed. Flesh of each cultivar was put in a plastic bag and kneaded by hand to homogenize the product.

Sensory evaluation

A group of 15–16 students and staff constituted the evaluation panel. Each plate had on it four different cultivars and one standard (which was a standardized canned product). The standard was given a score of 4 based on a scale from 1 to 7, dry to wet. Each panelist was instructed to evaluate the eight cultivars in their dry and wet mouthfeel in relation to the standard. All the samples except for the standard were coded to eliminate any bias towards cultivar names. The sum of scores of each cultivar was divided by the number of panelists to obtain the mean score.

Objective evaluation

The objective evaluation was done with a Haake Rotovisco Model RV-1 Viscometer. The rotor used was a SVP-II specifically designed to minimize separation of suspended particles. Baked sweet potato samples were mixed with three parts water to four parts sample (by weight) except for the initial test date of Oct. 10 when the sweet potato flesh to water ratio was 1:1 by weight. It was found after the first test that the equal weight ratio tended to narrow the range of the scale reading and, hence, a weight ratio of 1:0.75 (sweet potato to water) was used for the later test dates. The baked sweet potato flesh was mixed with water using a glass rod as stirrer. The batter was allowed to stand covered for 4 hr prior to testing to reach equilibrium conditions. Each variety was tested at all the 10 available rotor speeds with six replications. All the tests were conducted at 25°C, the temperature being maintained constant by a circulating water bath. The choice of this temperature had no particular significance except that it was about the room temperature.

Six samples from each cultivar were taken for the experiment. The first sample was subjected to the 10 rotor speeds beginning from the lowest and ascending stepwise to the highest rpm. Then the rpm was reduced to the next lower one and so on until the lowest. The two readings obtained, one while going from low to high and the other going from high to low, were averaged. The second sample was also treated the same way. The third and fourth samples were initially subjected to the highest rpm and proceeding gradually until the lowest and then again increasing the rpm to the highest by stages. Again the two readings were averaged for each sample. The fifth sample followed the same pattern as that of the first except for starting from the middle and completing the cycle in the mid-section of the range of rpm. The sixth sample followed the same procedure as that of fifth in the reverse order, beginning in the middle and proceeding downward to the lowest rpm and then ascending gradually to the highest and again descending to the middle. The above mentioned procedure was followed to average the effects of shearing; however, it was found that there were no major differences in the scale readings. A total of six observations for the 10 rpm for each variety culminated in 480 data points for each test date. Sample data are shown in Table 1. A complete record of the data are presented in Rao (1974).

RESULTS & DISCUSSIONS

THE DATA were analyzed first to determine if there were any significant differences between varieties for each test date. The ratio of shear stress in dynes/cm^2 to apparent shear rate in 1/sec yielded the apparent viscosity in poise. Note that the values obtained for shear rate are termed apparent because the fluid was non-Newtonian (Van Wazer et al., 1963). The sweet potato purees exhibited a pseudoplastic behavior with yield stress. A complete discussion and analysis pertaining to the mathematical model and determination of true shear rates etc. are presented by Rao (1974). It should be emphasized here that the apparent viscosities are not constant for a non-Newtonian fluid and vary with the apparent shear rates. Having obtained the apparent viscosity for each rotor speed, an analysis of variance was performed to obtain significant differences in apparent viscosity between the cultivars at each rpm for each test date. A summarized analysis of variance for one

Table 1—Data of Pelican Processor for Oct. 10, 1973

Apparent shear rate (sec^{-1})	Shear stress[a] (dyne cm^{-2})					
γ	τ_1	τ_2	τ_3	τ_4	τ_5	τ_6
462.00	2835	3314	3129	3314	3277	2872
231.00	2319	2356	2798	2577	2651	2762
154.00	2062	2099	2467	2283	2283	2504
77.00	1767	1804	2136	1952	1988	2209
51.33	1620	1657	1952	1804	1767	1988
25.67	1473	1510	1767	1657	1620	1841
17.11	1399	1399	1657	1620	1583	1694
8.56	1326	1289	1583	1546	1436	1583
5.70	1326	1252	1510	1510	1400	1546
2.85	1326	1178	1436	1436	1289	1510

[a] Each column of $\tau_1, \tau_2 \ldots \tau_6$ denotes a different sample (replication) and is the average of two observations.

Table 2—Means and analysis of variance of apparent viscosity of the cultivars at different storage dates at 97.2 rpm

Cultivar	Mean Viscosity (centipoise)				
	Oct. 10[a]	Oct. 17	Oct. 30	Nov. 14	Dec. 12
Pelican Processor	2566	2893	2534	2550	2048
Numbered	1857	2311	1937	1172	1618
Australian Canner	1275	2574	1777	1004	1148
Jewel	1116	1634	1028	1490	1482
Centennial	996	1642	1403	1212	1028
Nugget	813	1865	1028	1172	1753
Gem	773	948	829	534	948
Porto Rico	725	1140	1092	933	964
F Value	87.06	57.51	81.19	74.91	25.19
Probability > F	0.0001	0.0001	0.0001	0.0001	0.0001
LSD at 1%	263	343	237	269	314
LSD at 5%	196	256	178	201	235

[a] The tests on Oct. 10 were performed on uncured cultivars with sweet potato to water ratio = 1:1 by weight, while tests on all other dates were performed on cured cultivars with sweet potato to water ratio = 1:0.75 by weight.

Table 3—Comparison of correlation coefficients between taste panel and apparent viscosity and logarithm of apparent viscosity[a]

Date	Taste panel versus	rpm			
		3.6	21.6	97.2	583.2
Oct. 10	Viscosity	0.64	0.65	0.66	0.69
	Ln viscosity	0.71	0.72	0.71	0.77
Oct. 17	Viscosity	0.88	0.88	0.86	0.87
	Ln viscosity	0.92	0.93	0.89	0.89
Oct. 30	Viscosity	0.83	0.83	0.83	0.82
	Ln viscosity	0.88	0.86	0.85	0.83
Nov. 14	Viscosity	0.85	0.85	0.82	0.82
	Ln viscosity	0.93	0.92	0.87	0.88
Dec. 12	Viscosity	0.81	0.82	0.83	0.83
	Ln viscosity	0.82	0.83	0.84	0.86

[a] In every case the significant level was equal to or less than 0.0001.

rotor speed is given in Table 2. It was found that there were significant differences between varieties at each speed on all test dates. In fact, the probability of having significant differences exceeded 0.99 for all the tests which, considering the biological nature of the specimens, is excellent.

The taste panel data for each date were then paired with apparent viscosity values for the same date and a regression analysis performed to determine the correlation coefficient between taste panel score and apparent viscosity. Figure 1 shows the relationship of taste panel scores and apparent viscosity for Oct. 17 and Dec. 12. It was found from the analysis of regression data that the linear correlation coefficient between taste panel and apparent viscosity ranged from 0.8–0.9. In other words, 64–81% of the variation in taste panel scores can be attributed to apparent viscosity. One should realize that the values 0.8–0.9 are only for linear correlation and, hence, provide only the minimum value of correlation. Correlation coefficients increased when the data were analyzed considering taste panel scores and logarithm of apparent viscosity (Table 3). It may be concluded that there definitely does exist a relationship between taste panel scores and apparent viscosity.

Considering the effect of aging on the cultivars, Figure 2 indicates the general trend. It is possible to divide the entire range of apparent viscosities into zones of dry, intermediate dry, intermediate moist and moist as related to mouthfeel characteristics. Figure 2 has been divided into boundaries classifying these zones; however, one should realize that these are relative to each other and taste panel. It was found that in general the cultivars tend to become more moist, i.e., the apparent viscosity decreases with increasing storage. However, it is interesting to note a few exceptions to this trend. Nugget, for example, started as quite dry, dropped to quite moist in 25 days, and then ascended to dry again on the last test date. This anomaly possibly is due to large variation between potatoes which seems to be characteristic of this cultivar (Nelson, 1973). Australian Canner started as very dry and descended to medium wet, thus showing the greatest change. The Pelican Processor remained the most dry while Porto Rico and Gem remained the most moist throughout. The other three cultivars, viz. numbered, Centennial and Jewel, more or less remained in their respective positions with respect to the other varieties. One may conclude that in general the cultivars tend to become moist, thereby narrowing their range and, hence, the differences between the cultivars decrease with increase in storage time.

It can be seen from the results (Table 3) that it is not necessary to conduct the

tests at several different speeds but one particular speed should suffice. The criterion for choosing that speed was based on the coefficient of variation of apparent viscosity at a given speed, the significant differences that can be obtained between varieties at that speed, and the correlation between apparent viscosity and taste panel for that speed. Table 4 gives coefficients of variation of apparent viscosity readings on Oct. 10 and Dec. 12 for the highest and lowest speed. It may be concluded that the higher speed has lower coefficients of variation compared to those at the lower speed. This in general was found to be true for all the test dates with a few exceptions in the cultivars.

Figure 3 indicates the behavior of all the eight varieties on Oct. 17. It may be inferred from Figure 3 that low speed seems to separate the apparent viscosities of the cultivars better than high speed. This phenomenon was observed to be true for all the test dates. Considering the taste panel and apparent viscosity regression, in general, there seemed to be no definite correlation between speed and correlation coefficient. It was possible to conclude that one should choose a speed that would avoid greater scatter at the lower values at the same time noting that the differences between the cultivars were more pronounced at those points. Hence, a rotor speed in the mid-range of the available speeds would provide the best results.

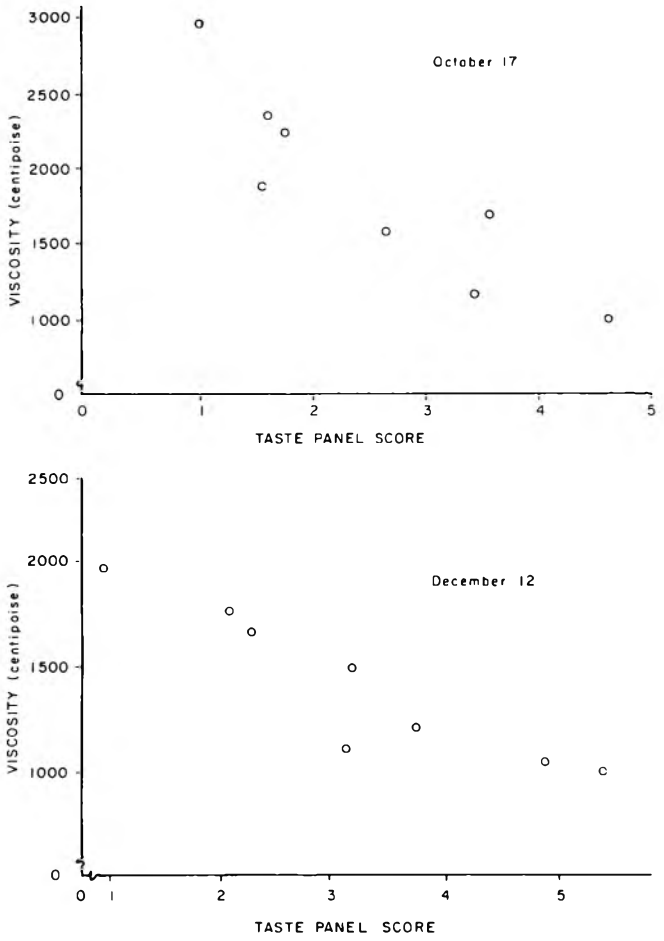

Fig. 1–Apparent viscosity (97.2 rpm) of sweet potatoes vs taste panel score for two dates.

SUMMARY & CONCLUSIONS

THE APPARENT VISCOSITY was a definite means of classifying sweet potato cultivars on the basis of moist mouthfeel quality for both uncured and cured specimens. The apparent viscosity in general tends to decrease with increase in storage time. Increased moistness was also observed in the taste panel scores, thereby increasing the reliability of viscosity measurements. The correlation of taste panel scores and apparent viscosity was positive and it was possible to define an arbitrary range of numerical values of ap-

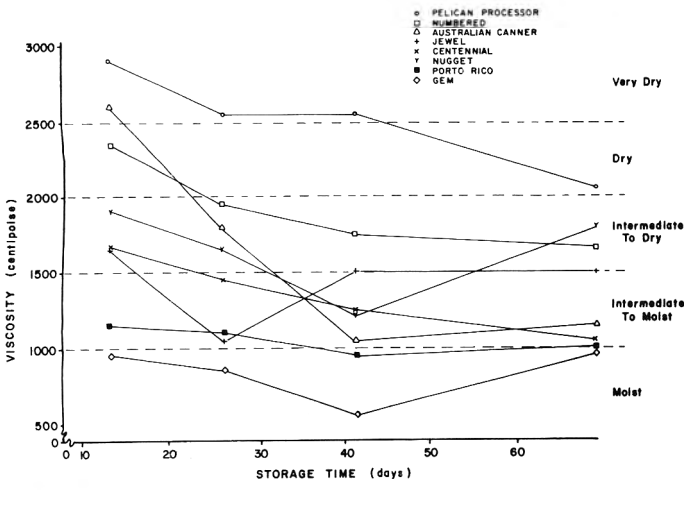

Fig. 2–Apparent viscosity (97.2 rpm) of sweet potatoes as a function of storage time.

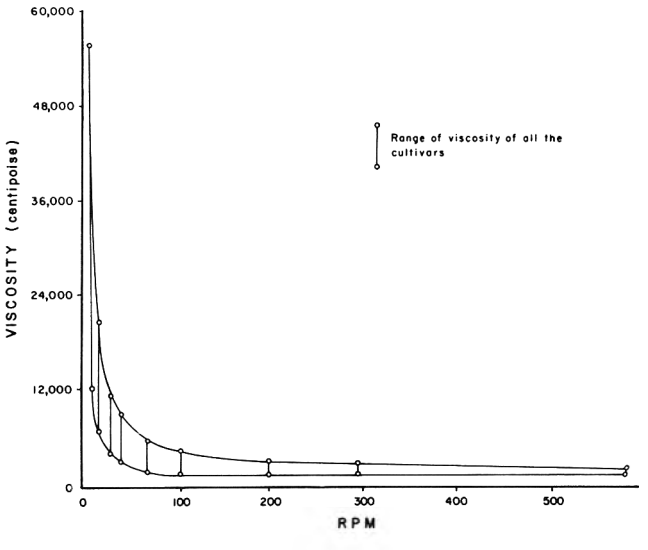

Fig. 3–Apparent viscosity of sweet potatoes as a function of rpm for Oct 17.

Table 4—Descriptive statistics for variable apparent viscosity on Oct. 10 and Dec. 12[a]

Date	Cultivar	3.6 rpm Mean (centipoise)	3.6 rpm Coefficient of variation (%)	583.2 rpm Mean (centipoise)	583.2 rpm Coefficient of variation (%)
Oct. 10	Pelican Processor	47770	8.88	676	7.05
	Number	32707	16.69	485	10.52
	Australian Canner	19366	25.65	383	19.32
	Jewel	16139	8.39	286	5.42
	Centennial	12911	6.33	262	2.99
	Nugget	10760	30.04	263	11.81
	Gem	10329	0.00	215	4.06
	Porto Rico	8822	5.97	203	2.15
Dec. 12	Pelican Processor	35720	24.48	582	17.39
	Number	23670	7.45	418	9.00
	Australian Canner	14202	0.00	351	11.32
	Jewel	22164	5.73	426	3.49
	Centennial	12696	11.89	334	7.00
	Nugget	26683	10.00	483	6.40
	Gem	14417	22.95	296	18.28
	Porto Rico	9683	7.30	306	7.31

[a] The tests on Oct. 10 were performed on uncured cultivars with sweet potato to water ratio = 1:1 by weight, while tests on Dec. 12 were performed on cured cultivars with sweet potato to water ratio = 1:0.75 by weight.

parent viscosity to classify dry, medium and moist mouthfeel characteristics. As an example, considering Figure 2 it is possible to define very dry as 2000 cp and above, intermediate to dry as 1500–2000 cp, intermediate to moist as between 1000–1500 cp and very moist as below 1000 cp. Finally, it is sufficient to test at only one rotor speed (angular velocity). Actually, all speeds used were suitable but those from 60–200 rpm (5–25 rad/sec) gave the best results.

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DIGESTIBILITY AND SAFETY OF LIMED HIDE COLLAGEN IN RAT FEEDING EXPERIMENTS

INTRODUCTION

CONSIDERABLE QUANTITIES of collagen are available each year in the form of limed cattlehide parts unsuited to the manufacture of leather. Collagen obtained from the corium layer of split hides after neutralization and washing (Mellon and Korn, 1956; Deasy, 1959), is essentially pure except for inherent fat and ash. This protein has unusual chemical and physical properties including a unique fibrous structure not found in other natural proteins. The fact that collagen can be dispersed under a variety of conditions to produce preparations ranging from a stiff fibrous paste to a liquid virtually free of fibers, or to a gel which resists hot water (Whitmore et al., 1972), suggests that its binding and texturizing properties may have uses in food or feed. Possible applications of these properties are now being investigated in the food and feed industries. At present, commercial use is limited to the manufacture of sausage casings which consumes only about 0.5% of the potential supply. The development of new uses, while not dependent on the nutritional qualities of collagen, requires knowledge of those qualities as measured by standards normally applied to food ingredients. Collagen represents about one-third of the total body protein (Gustavson, 1956) and does appear in various meat cuts. The present report does not deal with this native collagen per se, but with previously limed cattlehide collagen. Although differences between limed and unlimed or native collagen do exist (Veis, 1967), inferences may be made which will reflect on the nutritive and digestive properties of the native carcass collagen. While the digestibility of collagen has been investigated to some extent, partly because of its resistance to isolated proteolytic enzymes (Banga, 1965; Lamphiaho, 1966), and, also, due to its resistance to enzymes under physiological conditions, native fibrous collagen has often been regarded as indigestible (Grassmann, 1966; Ryan and Woessner, 1971; Cassel and Kanagy, 1949). Its low nutritional value as a protein in the form of gelatin was reported by Chapman et al. (1959) and Rama Rao et al. (1964), among others, and referred to by Stainsby and Ward (1969). Except in investigations of gelatin and soluble collagen as allergens (Maurer, 1954 and others), no reports of in vivo studies for toxicity have been found in the literature. This study was undertaken to fill this need for additional information on the digestibility and toxicity of limed cattlehide collagen. This paper reports the results of experiments in which collagen was fed to rats as an adjunct to the complete diet.

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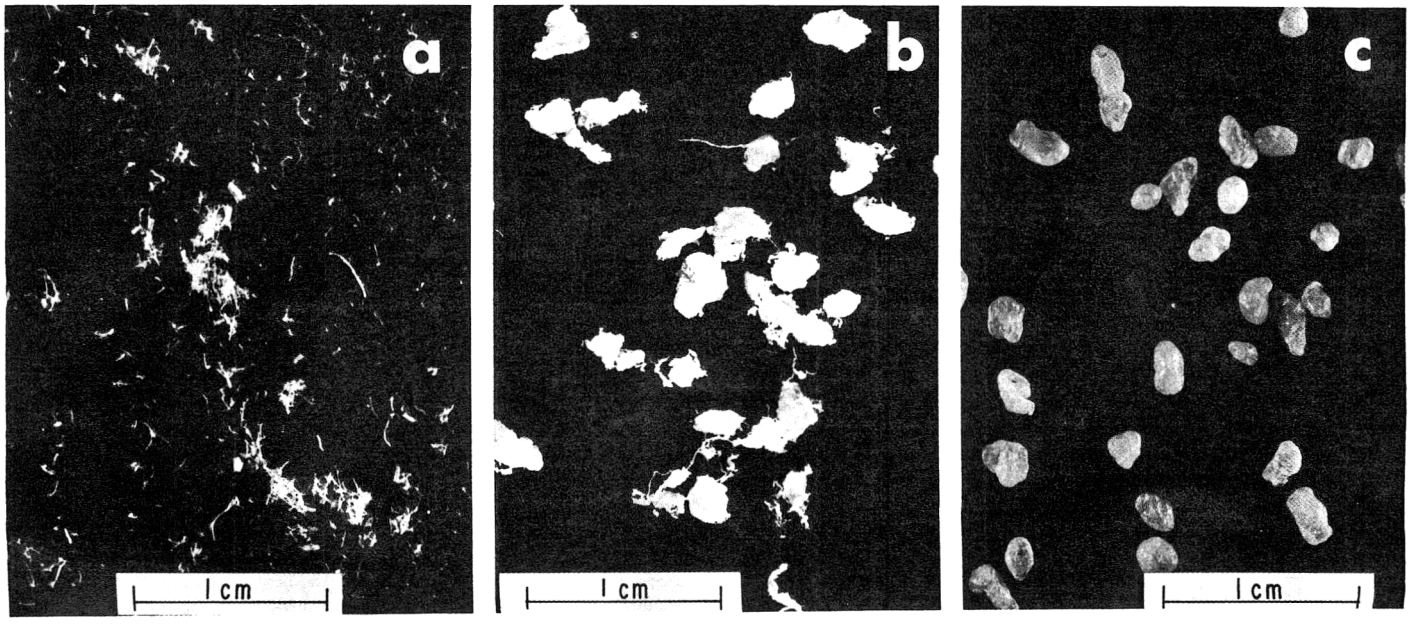

Fig. 1—Three types of collagen fed to rats. (a) Freeze-dried collagen, reground with dry ice in a cold Wiley mill to pass a 2 mm screen; (b) Particles produced by acetone dehydration of wet collagen cut through an 0.060 in. cutting head in an Urschel mill; (c) Particles produced by air-drying of wet collagen cut through an 0.060 in. cutting head in an Urschel mill. (Magnified 2.6X)

Table 1–Relative caloric assay on hide collagen vs. casein

Supplements added to purified basal diet[a]		Body weight changes (Avg per rat)		Moisture-free fecal wt/rat (g)
	g/rat/day	in 7 days (g)	Net gain (g)	
None	0	+1.2	–	0.64
Casein	1.0	+10.8	+9.6	0.58
Collagen	1.0	+9.5	+8.3	0.62

[a]Each rat received 5g of purified basal diet per day, plus supplement indicated.

EXPERIMENTAL

Preparation and characteristics of collagen

Hide collagen from fresh limed cattlehide splits was prepared by a deliming, neutralizing treatment followed by thorough washing (Whitmore et al., 1970). The splits were then cut while frozen to obtain hide particles which passed a 10 mm perforated screen.

For rat feeding tests the particles were freeze dried in shallow pans resting on platens warmed to 57°C in a vacuum <3 cm Hg. The dehydration required about 8 hr and was repeated on several lots to make a single composite of about 15 kg. The lyophilized material was further ground dry in a cold Wiley mill with added dry ice to pass a 2 mm screen (Fig. 1a). This product was found to be 76.2% collagen based on Kjeldahl nitrogen, 10.8% moisture, 9.8% fat, 0.7% fiber and 0.4% ash. A water suspension had a pH of 5–6. No odor or taste was evident in this preparation. To estimate the gelatinization resulting from cutting, soluble material was eluted from 6g of 10 mm screened and lyophilized material with 40°C water at a rate of 200 ml/hr; 100 ml aliquots of extract were dried at 95°C and weighed. The three aliquots taken in the first hour accounted for about 87% of the solids extractable in 4 hr. The extracts in the last hour accounted for only 3% of the total extracted. The total solids extracted in 4 hr were found to be between 12.2 and 12.8% of the starting material on a dry basis. When the analysis was performed on material from the same source that was ground in the dry state to pass a 2 mm screen, the warm water extraction produced a 12.8% loss. This degradation from the cold dry cutting was not regarded as an important difference from the degradation resulting from cutting wet frozen material followed by lyophilization at 57°C.

For study of the effects of preparative techniques on caloric availability and digestibility, two other types of comminuted hide were prepared. Both were cut wet and cold (30% collagen dry basis) using an Urschel Comitrol fitted with a nominal 0.060 in. head (Elias et al., 1970). The resulting granules were 1–2 mm in largest dimension and quite easily separable. Only about 7% of the product was extracted with 40°C water under the conditions described above. One lot of this comminuted hide was extracted with 2 volumes of acetone for 1 hr, the extraction repeated four times, and dried slowly at 25°C while covered to prevent water condensation. The product was essentially fat free white granules (Fig. 1b). A second lot was dried in air by slowly tumbling in a stream of warm air. Some aggregation of granules occurred and these were separated from the single granules by screening. The product was about 3% fat on a dry basis, smooth rounded light tan colored granules (Fig. 1c). These wet-cut granules, dried in the forms shown in Figure 1 and described above, were fed only as separate caloric availability and digestibility supplements.

Determination of digestibility and caloric availability

To a basal diet calorically restricted and fed at near maintenance levels, described by Rice et al. (1957), collagen or casein were added as the only proteins. Rats were fed in separate cages 5g of basal diet plus 1g of supplement each day for 7 days. Body weights before and after the test period were used to compare caloric availability of the two proteins after adjustment for the weight changes in the controls. In further tests, the level of supplement was raised to 2g/rat/day. Acetone dried (defatted) collagen was compared with air dried (not defatted), and the effect of dry cutting and particle size on digestibility was tested. Fecal weights of rats fed control and supplemented diets were compared to estimate digestibility. Weight gains of the test animals were used to estimate caloric availability.

Determination of toxicity

The collagen was mixed with a commercial rat diet at a level of 20%, fed ad lib to weanling (50g average) males and females. Casein added at the same level and the diet alone were used as controls. Groups of five rats of each sex were fed on each diet. During the 90-day feeding period, weekly body and feed consumption records were maintained. Urinalysis and hematology studies were made during the last 10 days of the test period. At the end of the feeding period body and organ weights were compared as were histological findings.

RESULTS

Digestibility and caloric availability

Table 1 shows results of a caloric availability test where the collagen supplement was added to a purified diet which was fed at a rate (5g/rat/day) to restrict caloric intake to a maintenance level. The weight gains shown by collagen-fed rats were 86% those of rats fed equal daily supplements of casein. Fecal weights also indicated the digestibility of the fibrous collagen at close to 100%.

In another test comparing caloric value of the same collagen with that of gelatin (Nutritional Biochemicals Corp., Cleveland), the weight gains indicated that 1g of collagen was equivalent to 1.5g of gelatin as an energy source.

In a subsequent test, 2g supplements per rat per day, 29% of the total diet, were fed (Table 2). A comparison was made of caloric availability between gelatin, 2 mm dry ground collagen (3), 0.060 in. acetone dried defatted (4), and 0.060 in. air dried collagen (5). (3, 4, 5 refer to Table 2 notation and to a, b, c in Fig. 1). Results at this level of supplementation show collagen 3 to be about 90% digestible and slightly lower in caloric availability than gelatin. Hide collagen cut wet and cold at 1–2 mm maximum dimension followed by acetone dehydration (4) or air drying (5) was compared to study the effect of fat or physical state on caloric availability. To minimize possible pseudo-weight gains due to undigestible intestinal residues, all rats were fed basal diet only (5g/rat/day) for a period of 48 hr following the 7-day period of supplementation. Fecal weights per se could not be obtained due to diarrhea, hence combined urine and feces weights were used. Since collagen (3) contained 10% fat, collagen (4) was essentially free of fat and collagen (5) contained about 3% on dry basis, the results indicate that within the range tested, fat content is not an important consideration in digestibility or caloric availability of fibrous collagen when it is fed to rats. It also appears that the degree of fiber development in a palpable or chewable size range does not affect digestibility in rats.

Toxicity

Body weights and organ weights (Table 3) of rats fed the 2 mm dry ground collagen for 90 days were comparable to those of rats fed casein except for the kidney weights. In both sexes the kidneys were significantly heavier. The importance of this effect is minimized, since the microscopic examination of kidney tissue sections did not show any differences from comparable tissues of control animals. The results of the blood and urine analyses suggest that the only significant effect was an increase in hemoglobin concentration in the female rats fed casein and collagen. It was noted that during the collection of blood samples the rats fed collagen were more sensitive to ether anaesthesia, and the blood appeared to clot more rapidly. Pathological findings from studies of the stomach, small and large intestine, heart, trachea or larynx, lung, pancreas, liver, kidneys, urinary bladder, spleen, pituitary, thyroid (sometimes with parathyroid), adrenals, testes or ovaries, uterus in females (sometimes seminal vesicles in males), thymus and brain (sagittal section) indicated no significant lesions or differences between

the control and collagen-fed rats.

Footnote (b) to Table 2 notes that some rats in each group had diarrhea. Collagen and gelatin were fed at 2g/rat/day added to 5g basal diet in this test. This is at a rate of 28+% of the total diet, considerably higher than the 20% fed for 90 days for toxicity studies, and several times the level proposed for food texturizers. The diarrhea may be linked to the higher hemoglobin levels in the blood of some rats. This in turn may be linked to the susceptibility to ether anesthesia after 80 days on the diet. Since the diet was fed dry, an assumption was made that the water-holding capacity of the collagen might be simply dehydrating the animals. However, careful checks of water intake between test and control animals revealed no significant differences. The cause and effect of these findings should be the basis for some future collagen feeding tests.

DISCUSSION

Digestibility

Studies of the digestibility of collagen by isolated enzymes have been reported in the literature. Mandl (1961) has reviewed the effects of temperature, acids, swelling, liming, grinding etc. on the susceptibility of collagen to attack by trypsin. Woessner (1968) has more recently reviewed the proteolytic digestion of collagen under physiological conditions of pH, temperature and ionic strength. Much of the literature is not relevant to our findings because it is concerned with effects on soluble or reconstituted collagen rather than on native, insoluble or previously limed "matrix" collagen (Veis, 1967). The reasons for feeding the dried granular hide collagen in parallel with the dry cut or fibered form were to establish some limits, if any exist, to the degree of fiber separation required for gastric and alimentary digestion. The air-dried granules were at the upper limits of size which permit collagen incorporation in food mixtures without adding a gristle texture which is objectionable. The digestibility of these granules at 29% dietary supplementation (Table 2) either with or without inherent fat indicates that collagen need not be reduced to fibers before ingestion. The finding of 90 rather than

Table 2—Rat caloric assay and digestibility of collagen samples compared to gelatin[a]

Supplement		Avg wt gain/rat 7 days (g)	Avg wt change/rat on basal for 2 days (g)	Net change/rat Columns 2 + 3 (g)	Net change/rat Minus basal (g)	Urine and fecal output/rat/ 9 days MFB (g)	Apparent digestibility[e] (%)
(1)	Basal control	2.4	0	+2.4	–	4.0	100
(2)	Gelatin	20.2[c]	–3.6	+16.6	+14.2	5.0	93
(3)	Collagen[b] (2 mm 10% fat)	19.8[c]	–4.0	+15.8	+13.4	5.4	90
(4)	Collagen[b] (acetone dried 0.060 in. fat free)	15.4[c]	–2.8	+12.6	+11.4[d]	5.4	90
(5)	Collagen[b] (air dried 0.060 in., 3% fat)	18.0[c]	–4.2	+13.8	+11.4	5.4	90

[a] 5 Male rats/group, Sprague-Dawley strain, 4 wk of age
[b] Numbers 3, 4, 5 refer to a, b, c in Fig. 1; the 3 collagens and gelatin were fed at a level of 2 g/rat/day as supplements to the basal diet (5 g/rat/day).
[c] Some rats in each group had diarrhea.
[d] Corrected for rejected supplement by 2 rats
[e] Apparent % digestibility = [(supplement intake − increase in urine and feces)/supplement intake] X 100
Example: Collagen (3) $\frac{[14g - (5.4g - 4g)]}{14g} \times 100 = \frac{12.6}{14} \times 100 = 90\%$

Table 3—Terminal body and organ weights of rats fed a 20% collagen diet for 90 days[a]

Diet No.	Diet Supplement	Sex	Body wt (g)	Organ weights in grams per 100 grams body weight[b] Liver	Kidneys	Spleen	Heart
1	Purina basal	M	477 ± 50	3.47 ± 0.21	0.67 ± 0.07	0.14 ± 0.01	0.29 ± 0.04
2	+20% Casein	M	454 ± 36	3.82 ± 0.39	0.70 ± 0.06	0.15 ± 0.03	0.28 ± 0.02
3	+20% Collagen	M	440 ± 61	3.44 ± 0.27	0.81 ± 0.09*	0.15 ± 0.01	0.31 ± 0.03
4	Purina basal	F	281 ± 17	3.57 ± 0.18	0.67 ± 0.05	0.19 ± 0.01	0.32 ± 0.03
5	+20% Casein	F	295 ± 14	3.33 ± 0.20	0.72 ± 0.02	0.19 ± 0.03	0.35 ± 0.03
6	+20% Collagen	F	280 ± 14	3.52 ± 0.13	0.83 ± 0.05**	0.18 ± 0.02	0.34 ± 0.02

[a] Mean starting weight = 49g, male; 50g, female (Sprague-Dawley strain). Tr al duration = 90 days. One male rat from group 3 died during blood sampling (from ether anesthesia) at day 80 of feeding trial.
[b] Adrenal, thyroid and testes weights (not tabulated) were not significantly different.
*$P < 0.05$
**$P < 0.01$

100% digestion of collagen in the rat gut is probably related to the 10–20% unconverted residue usually found in commercial gelatin manufacture.

Toxicity

The lack of sub-acute toxicity in rats fed diets containing up to 20% collagen for 90 days was not surprising, but confirmation of a long standing assumption. The significantly higher kidney weights in rats fed collagen over those fed casein supplement may be attributable to the higher nitrogen content of the collagen. The kidney weight difference was 12–14%. Collagen is 18.6% N_2, casein is 15.9%—a difference of 15–17%. A functional hypertrophy of the kidney is known to occur in rats fed high protein diets (Osborne, 1926–1927).

CONCLUSIONS

DELIMED, washed, fibrous, insoluble hide collagen when fed to rats is well digested (90%) and serves as a source of energy. It is not toxic when fed at a high percentage of the diet for relatively long periods.

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EVALUATION OF LACTOSE AS A TRANSFER CARRIER FOR VOLATILE FLAVOR CONSTITUENTS

INTRODUCTION

AS PART OF a continuing study concerned with minimizing the formation of artifacts and changes in the relative amounts of volatiles in essences prepared for analysis, the porous polymers for trapping volatile constituents from carrier gas streams were explored. It had been established that Porapak Q could be used to effectively trap volatiles, which can be recovered from the polymer without obvious changes in the aroma properties (Jennings et al., 1972; Tressl and Jennings, 1972). This suggested the possibility that natural flavor constituents, frequently lost in processing operations, might be trapped on Porapak Q or some similar material, and subsequently transferred to a food-grade carrier for the re-addition of these natural flavor constituents to the processed food. Because of its low level of sweetness, high adsorptivity and ready availability, lactose was selected as a suitable carrier. Specifically, we were interested in establishing desirable parameters for transferring flavor volatiles to lactose, and evaluating variables that influenced the ability of lactose to maintain this flavor in storage. In both cases it was highly desirable to compare the instrumental analyses with sensory evaluations. Therefore, representative aliquots of each sample of flavor-impregnated lactose, in dry form and dispersed in water, were subjected to sensory analysis and procedures were established for re-isolating the lactose-adsorbed volatiles so that they could be examined by gas chromatography (GC).

MATERIALS & METHODS

Lactose

Because of its higher adsorptive powers, freshly prepared anhydrous α-lactose was used as the transfer agent. At 130°C and under high vacuum, lactose evenly spread on a Petri plate lost 85% of the calculated weight of water of hydration in 5 hr, and over 99% in 24 hr. Lactose (obtained from Fischer Scientific Co.) was heated at 130°C for 24 hr under high vacuum, and stored in sealed containers. Visual examination of the lactose powder under the optical microscope at 1000X showed irregular-shaped particles varying in size by approximately 200-fold. Total surface area was not determined.

Flavor source

Valerie bananas were chosen as a source material, because of their strong characteristic aroma and ready availability. 2 kg of bananas were placed in a desiccator and swept with purified air as described in an earlier publication (Tressl and Jennings, 1972).

Porapak Q

2g of 100–120 mesh Porapak Q (Waters Associates) was placed between glass wool plugs in a 12 mm glass tube ca. 7 cm long. N_2, purified by passage through freshly regenerated molecular sieve (5A and 13X), was passed, in a forward direction, through the trap at a rate of 120 ml/min at 100–120°C for 24 hr (Jennings et al., 1974a). Collections were made on the traps in this same direction for the indicated periods utilizing purified air at 120 ml/min as the carrier. The Porapak was maintained at 26°C during trapping, and developed 30 min with purified nitrogen at 120 ml/min at this same temperature to remove water (Tressl and Jennings, 1972). Recovery was accomplished by backflushing at 100°C for 30 min to glass tubes tightly packed with 5g of lactose. The tubes were then flame-sealed and stored at −10°C. When the tubes were opened later, the lactose was thoroughly mixed before aliquots were taken for analyses.

Gas chromatography

Quantitative studies were made with a Hewlett Packard 5700, adapted to glass inlet splitters of our design and manufacture (Jennings, 1974) and fitted with glass capillary columns, 0.25 mm × 88m, coated with Carbowax 20 M admixed with 1–5% Igepal CO 880 or benzyl triphenyl phosphonium chloride as an anti-tailing additive (Jennings and Wohleb, 1974; Jennings et al., 1974b). 0.5 µl injections were used, with a split ratio of ca. 70/1. The injector and detector temperatures were maintained at 225°C and the column was maintained isothermally at 100°C. The output signal was fed through an Infotronics 208 digital integrator to the recorder.

Instrumental analyses

A variety of solvents–including methanol, benzene, cyclohexane, pentane, carbon disulfide, carbon tetrachloride, chloroform, methylene chloride, ether and acetone–were evaluated for the efficiency and reproducibility with which they recovered volatiles from the flavor-

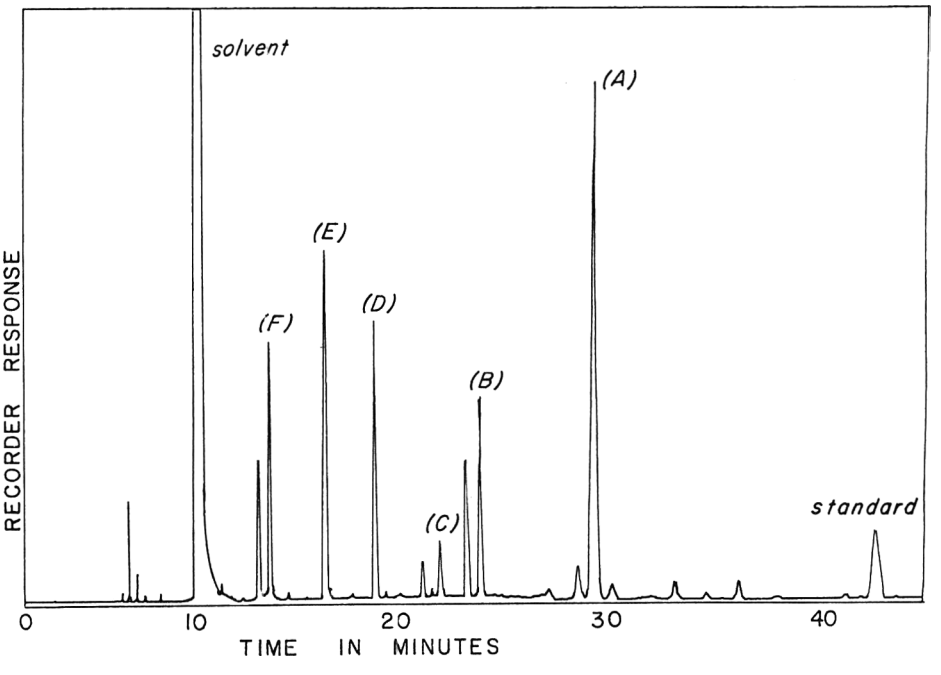

Fig. 1–Typical chromatogram of benzene-desorbed essence from flavor impregnated lactose.

impregnated lactose for gas chromatographic analyses. The procedure eventually adopted was as follows: 0.5g of volatile-impregnated lactose was placed in a glass weighing ampoule and covered with a minimum quantity (0.25 cc) of benzene. (In some later work, these quantities were changed to 1g lactose and 0.5 cc benzene). A small plug of glass wool was inserted, and the neck of the ampoule was drawn to a tapering point and flame-sealed. After 24 hr, the container was inverted, and the few μl of free solvent-containing desorbed volatiles filtered through the glass wool into the tapered neck, which was removed to obtain access to the sample.

Although a large number of volatiles have been identified as constituents of banana (e.g., Hultin and Proctor, 1962: McCarthy et al., 1963; Tressl and Drawert, 1973; Wick et al., 1969), only a few of the major components (see Table 2) were detectable in the unconcentrated benzene eluate. While solvent evaporation increased the number of detectable components, this also caused changes in the relative ratios of the individual volatiles. To avoid this complication, the study was limited to quantitating six major constituents.

Table 1—Sensory terminology and references used in description of banana aroma

1.	Overall aroma intensity	
2.	Banana references[a]	
		Green pulp
		Ripe pulp
		Overripe pulp
		Green skin
		Ripe skin
		Overripe skin
		Green pulp, cooked
		Ripe pulp, cooked
		Overripe pulp, cooked
		Green skin, cooked
		Ripe skin, cooked
		Overripe skin, cooked
3.	Other terms and references[a]	
		Artificial banana[b]
		Banana chips, commercial
		Banana pudding (Brand H)
		Banana pudding (Brand D)
		Artichoke, cooked
		Starchy (cornstarch:water, 3:1)
		Caramelized (20% sucrose soln, heated to light yellow)
		Scorched (20% sucrose soln, heated to light brown)
		Ethyl alcohol, 6%
		Isopropyl alcohol, 6%
4.	Additional terms[c]	
		Fruity
		Floral
		Perfumy
		Earthy
		Woody
		Grassy
		Cowy
		Medicinal
		Fishy
		Candy
		Sweet

[a] Samples present for comparison during evaluation of experimental samp es

[b] Mixture of iso-amyl acetate, iso-butyl acetate, iso-amyl alcohol, iso-butyl butyrate, n-butyl acetate and n-butyl butyrate (2:1:1:3/4:1/2:1/2, respectively).

[c] No reference samples provided

Transfer temperature

Constant back-pressure devices (exit via a variable liquid head) were used to simultaneously conduct four identical air streams from the banana chamber to four Porapak Q traps. The volatiles in these traps were subsequently transferred to lactose at lactose-trap temperatures of 26°C (room temperature), 0°C (wet ice), −24°C (ice-NaCl) and −80°C (dry ice).

Sensory analyses

Sensory analysis of the aroma characteristics were performed by seven departmental employees who had been selected and trained in the use of descriptive sensory evaluation, using bananas and compounds isolated from bananas as test materials. Experimental samples of lactose impregnated with banana volatiles were evaluated "dry" (0.5g lactose in a 60-ml glass container) and dispersed in distilled water (0.5g lactose in 10 ml water in a 60-ml glass container). Using the terminology shown in Table 1, judges first familiarized themselves with all the odor reference samples which consisted of pulp and skin of bananas of different maturities, both cooked and uncooked, as well as examples of the other substances listed as "other references" on the evaluation sheet. Using a scale of 0 = none and 10 = very strong, odor intensities were assigned to each quality factor perceived, as well as to overall intensity.

In one experiment, aroma characteristics were evaluated for lactose samples representing trapping of volatiles for 1, 2, 3, 4, 5 and 6 consecutive days. For a second experiment, the samples evaluated consisted of lactose samples that had been used to trap volatiles at −80, −24, 0, and 26°C.

RESULTS & DISCUSSION

FIGURE 1 shows a chromatogram typical of the benzene-desorbate from flavor-impregnated lactose. Because this solvent exhibited the most efficient recovery of the volatiles from flavor-impregnated lactose among a variety of solvents as ascertained by running many triplicate trials, it was utilized throughout the study.

Table 2—Boiling points of major components

Code[a]	Compound	Boiling point (°C)
A	iso-amyl n-butyrate	179
B	n-butyl n-butyrate	167
C	iso-amyl alcohol	182
D	iso-butyl n-butyrate	157
E	iso-amyl acetate	142
F	n-butyl acetate	126
G	iso-butyl acetate	117

[a] From Figures 3 and 4

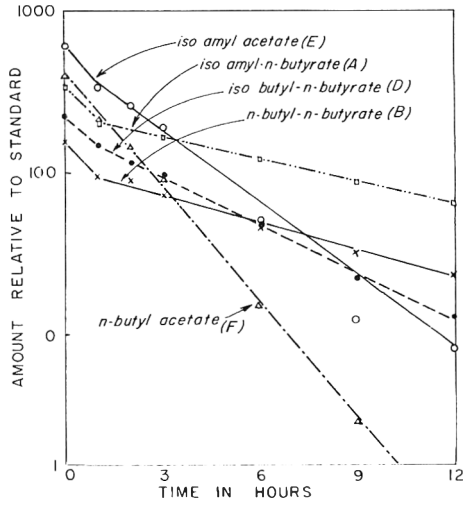

Fig. 2—Recovery of individual volatiles from lactose stored unsealed at room temperature for different periods.

When lactose was used directly as the trapping agent (i.e., without trapping initially on Porapak), the large amount of water caused it to become pasty, and the traps soon clogged. Additionally, preliminary "sniff-tests" indicated that the lactose impregnated with flavor by transfer from Porapak traps had a much stronger aroma than lactose used for the direct adsorption of banana volatiles. Apparently the amount of material recovered by the Porapak procedure exceeded the adsorptive capacity of the lactose. On exposed storage, the lactose lost flavor rapidly, and in a period of 1–2 days, the major flavor content had vanished (see below). In sealed containers, the stability was much better, even at room temperature. When stored in sealed containers at 4°C, the level of flavor appeared to remain fairly constant for at least several months.

To determine how effectively lactose retained volatiles which had been transferred from Porapak traps, a large lactose sample heavily charged with banana volatiles was mixed carefully to ensure uniformity and divided into nine 0.50-g portions. These were placed in extraction tubes and allowed to stand unsealed at atmospheric pressure and room temperature for 0–24 hr. Each sample was then extracted as described above, and the extract analyzed by GC. Figure 2 shows a semi-logarithmic plot of the loss of each component from lactose for periods up to 12 hr. For most compounds, the plots approximate straight lines except for the initial period, which is characterized by a more rapid rate of loss. This observation

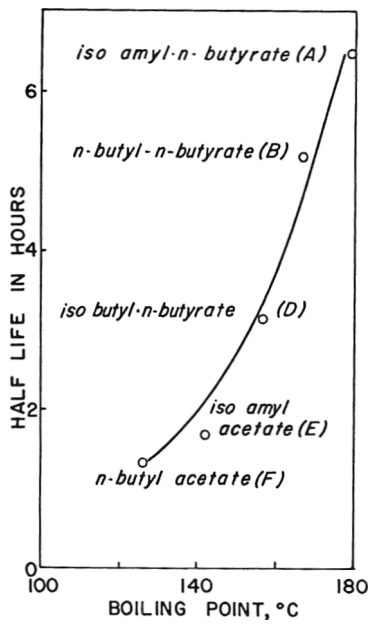

Fig. 3–Rate constants of desorption of individual volatiles as a function of their boiling points.

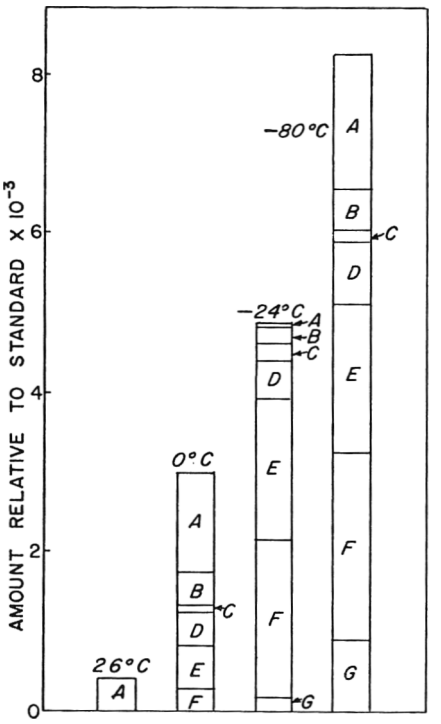

Fig. 4–Effect of lactose temperature on efficiency of volatile recovery.

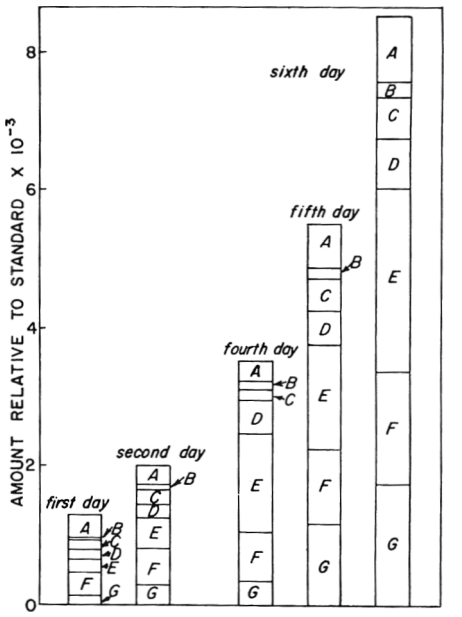

Fig. 5–Effect of ripeness on volatile recovery and volatile composition. Lactose temperature during transfer −80°C.

agrees with the conclusion of Gray and Roberts (1970).

At these high initial levels, the adsorptive capacity of the lactose probably had been exceeded, and these condensed but unadsorbed volatiles were initially lost at a rapid rate. The loss of the adsorbed volatiles then follows normal first-order kinetics. Figure 3 shows the half-life individual adsorbed volatiles as a function of their boiling points, indicating that the more volatile components are lost at a faster rate.

Figure 4 shows the effect of lactose temperature on the efficiency of recovery of volatiles transferred from Porapak Q traps, as determined by gas chromatographic analysis of benzene eluates from the lactose. Although each of the aliquots of lactose was exposed to the same quantity of the same volatiles under identical conditions of flow, the total amount of volatiles recovered at each temperature indicates that the efficiency of lactose in retaining the volatiles was inversely proportional to the lactose temperature (Fig. 4). There are variations in the relative amounts of individual volatiles in the −24°C sample that we are unable to explain.

Table 2 lists the boiling points of these major components of banana essence. It is evident that the lactose efficiency at the different temperatures (Fig. 4) is, in most cases, inversely proportional to the volatility of the compound; relatively larger amounts of lower boiling components were collected at lower lactose temperatures, and as the lactose temperature increased, the efficiency for individual components decreased with increasing volatility. Because this changes the rela-

Table 3–Correlation (r) between GC data (integrated peak area) and sensory data (aroma intensity values for overall odor, natural banana odor and artificial banana odor)

		GC peaks								
		A	B	C	D	E	F	G	A–G	A–D
Overall odor	Dry	0.93*	0.91*	0.92*	0.90*	0.93*	0.93*	0.95*	0.96**	0.97**
	Wet	0.50	0.85	0.45	0.92*	0.84	0.71	0.57	0.72	0.68
Natural banana odor	Dry	−0.44	−0.05	−0.47	0.14	−0.06	−0.27	−0.37	−0.22	−0.25
	Wet	0.24	0.22	−0.22	0.39	0.25	0.08	−0.11	0.07	−0.01
Artificial banana odor	Dry	0.96*	0.95*	0.93*	0.92*	0.96**	0.97**	0.97**	0.40	0.99***
	Wet	0.69	0.95*	0.63	0.96**	0.94*	0.86	0.73	0.86	0.82

* ** ***Significant correlation coefficients at the 5, 1 and 0.1% levels of probability, respectively.

tive amounts of individual volatiles, it could have a profound effect on the quality of the aroma.

Figure 5 indicates that under any one set of conditions, the Porapak-lactose system exhibited a high degree of fidelity in recovery of volatiles. The amount of each individual volatile showed a regular and progressive increase with increasing ripeness, but the relative amounts of each individual volatile were reasonably constant. This is consistent with the report of Tressl and Jennings (1973), bearing in mind that the 24-hr trapping periods employed in this study would average out the cyclic phenomenon shown by these authors. Even so, the over-ripe banana certainly exhibited an aroma that was quite different from a ripe or green-ripe banana, agreeing with previous investigators (McCarthy et al., 1963). Other minor volatiles not followed in this study may have contributed to this difference, or as the concentrations changed, certain individual volatiles or combinations of volatiles may surmount a threshhold, which would have a profound effect on the aroma.

Sensory analysis

Three major aroma characteristics were perceived by the sensory panel: "overall banana intensity," "natural banana odor" and "artificial banana odor." Table 3 lists the correlation coefficients between the integrated peak area of the GC analyses and the aroma intensities ascribed by the sensory panel, for the experiment in which days of trapping (ripening levels) were examined. Note that much higher relationships were obtained for the dry lactose samples than for the hydrated samples. Whereas the sensory characteristic called "artificial banana odor" was highly related to GC peak areas, natural banana odor was not. The highest correlations were obtained when the sensory responses for overall odor and for artificial banana odor were compared with the combination of peaks A through D.

Several trends were apparent from the results of the sensory analyses. The overall odor intensity and the intensity of artificial banana odor generally increased with progressive days of trapping, corresponding to ripening of the banana samples. Responses to natural banana odor, however, reached a maximum after 4 days of trapping, then dropped. Samples that were dispersed in water (designated as "wet" in Table 3) had higher odor intensities than did the dry samples.

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IMMOBILIZATION OF PAPAIN ON COLLAGEN AND THE USE OF COLLAGEN-PAPAIN MEMBRANES IN BEER CHILL-PROOFING

INTRODUCTION

RECONSTITUTED COLLAGEN has been found to be an excellent carrier for the immobilization of enzymes and whole microbial cells. Several publications from our laboratory have described the preparation and characterization of collagen-immobilized enzymes (Vieth et al., 1972; Venkatasubramanian et al., 1972; Saini et al., 1972; Constantinides et al., 1973; Vieth and Wang, 1973; Vieth and Venkatasubramanian, 1974; Venkatasubramanian et al., 1974b) and whole microbial cells (Vieth et al., 1973).

The use of a proteinaceous material, such as collagen, as a carrier matrix for enzyme immobilization raises the question whether its use has to be necessarily limited to nonproteolytic enzymes. To answer this question, we studied the immobilization of three proteolytic enzymes—papain, ficin and rennin. These three enzymes were all successfully immobilized on collagen. In this paper, we present the preparation and the properties of collagen-papain membrane complexes.

The literature contains a number of reports on the immobilization of papain. Axen and Ernback (1971) chemically linked papain to cyanogen halide-activited polysaccharide carriers. Attachment of papain to a collodion membrane was reported by Goldman and coworkers (1965). Covalent binding of papain to polymeric matrices was accomplished by Manecke and Gunzel (1967). Preparation of insoluble papain through cyanoethylated starch anthranilites was presented by Mehltretter and Weakley (1972). Goldstein (1973) bound papain to polyanionic and polycationic resins. Immobilization of papain on porous glass by adsorption (Messing, 1970) and by covalent binding (Weetall, 1973) have also been reported in the literature. Intermolecular cross-linking of papain by reacting it with glutaraldehyde in dilute aqueous solution was accomplished by Jansen and Olson (1969); the cross-linked enzyme formed a water-insoluble product which possessed 12–48% of the original proteolytic activity.

Unlike much of the work published in the literature, our primary interest in preparing an immobilized papain derivative was to explore the possibility of using it in chill-proofing of beer. The beer chill-proofing process consists of hydrolyzing residual proteins in beer which would otherwise precipitate and cloud the beverage upon cold storage. Free proteolytic enzymes such as papain and ficin are widely used at present for this purpose. While the free enzymes are sometimes partially inactivated if the beer is pasteurized, in most cases they remain with the beer. From the standpoint of future labelling requirements, it would be desirable to remove the enzymes from the beer. An immobilized enzyme process would provide the possibility of reusing the enzyme, obviating the necessity of listing papain in the product.

The literature contains but few references on chill-proofing of beer by means of immobilized proteolytic enzymes. Wildi and Boyce (1971) and Weinrich et al. (1971) have discussed chill-proofing of beverages using enzymes attached to polymeric carrier matrices. Similar work was reported by Witt et al. (1970). These authors used covalently cross-linked papain [by the procedure of Jansen and Olson, (1969)] to chill-proof beer in batch and semi-continuous operations. In the work reported here, the degree of chill-proofing obtained with collagen-papain membranes was significantly better than that obtained in the work of Witt et al. (1970).

EXPERIMENTAL

Materials

Collagen. Cow-hide collagen was obtained from the USDA Eastern Regional Research Laboratories, Philadelphia, Pa.

Papain. Two different preparations of papain from papaya latex were purchased from Worthington Biochemical Corp., Freehold, N.J. The specific activities of these preparations were 0.5 IU/mg protein and 12.7 IU/mg protein, respectively. (One international unit of papain activity hydrolyzes one micromole of benzoyl arginine ethyl ester per minute at 25°C.)

Substrates. 2% gelatin (Knox Gelatin Inc., Johnstown, N.Y.) was used as the high molecular weight substrate. Benzoyl-L-arginine ethyl ester (BAEE) purchased from Sigma Chemical Co., St. Louis, Mo., constituted the low molecular weight substrate.

Chemicals. All chemicals used were reagent grades.

Methods

Preparation of collagen-papain membranes by direct macromolecular complexation. The procedure for immobilizing enzymes on collagen by the method of complexation has been described in detail elsewhere (Vieth and Venkatasubramanian, 1974). 50g of shredded hide collagen was dispersed in distilled water using a Waring Blendor. The pH of the dispersion was lowered to 4.5 using concentrated lactic acid. 1g of papain (total activity 500 IU) was added to the dispersion and thoroughly mixed. The final dispersion containing 1% (w/v) solids was cast on a Mylar® sheet to form a membrane, using a Gardener knife. The membrane was left to dry at room temperature. The thickness of the dried membrane was 0.125 mm. The dried collagen-papain membrane was tanned by dipping in 10% (w/v) glutaraldehyde solution at pH 5.0 for 30 sec. The tanned membrane was then washed thoroughly in running tap water for 1 hr.

Covalent binding of papain to collagen. The method of Silman et al. (1966) was used to covalently link papain to collagen membrane. 5g of wet hide collagen pulp were made into a dispersion of pH 7.7 by addition of sodium phosphate buffer with good stirring. 0.555g of purified benzidine-2,2'-disulfonic acid, obtained from American Cyanamid Co., Bound Brook, N.J., were activated by addition of hydrochloric acid and sodium nitrite to form the diazonium slat. The diazonium salt was then added to the collagen dispersion and coupled thereto. The adduct was isolated and washed. Thus, reconstituted collagen was modified to a diazonium derivative prior to enzyme immobilization. 200 mg of papain, dissolved in 6 ml distilled water were added and linked to the modified collagen, which resulted in a fiber-mat type of structure on drying.

Preparation of collagen-papain biocatalytic modules. Spirally wound biocatalytic modules have proven to be excellent reactor configurations for the use of collagen-enzyme membrane systems (Vieth and Venkatasubramanian, 1974). Collagen-papain membranes were also tested in a spirally wound reactor. 15g of collagen-papain membrane were layered on Vexar® netting (E.I. duPont, Buffalo, N.Y.) which served as a backing material. It separated the successive layers of papain-collagen membrane, thus preventing overlapping of the membrane layers. A stainless steel rod, 6.25 mm diam was

used as a central core element. A spiral reactor configuration was formed by coiling alternate layers of the membrane and backing around the central spacer element. The spiral cartridge with an outside diameter of 5 cm was fitted into a Plexiglass outer shell 7.5 cm long. The plastic housing was affixed to two threaded aluminium end plates provided with an inlet and outlet for the flow of the substrate over the membrane surface. A uniform axial distribution of the substrate was achieved by metering the flow through a distributor plate containing a number of 0.5 mm diam holes.

Assay of immobilized papain. The biocatalytic module containing the immobilized enzyme was tested in a batch recycle reactor system which provides a rapid and convenient way of evaluating the catalytic potency of the immobilized enzyme (Vieth et al., 1972). A mini-fermenter (Fermentation Design, Philadelphia, Pa.) provided with temperature and agitation control was used as the substrate reservoir. The substrate was circulated through the reactor at a desired flow rate (usually 400–500 ml/min) by a diaphragm-type controlled volume pump (Model R121A, Milton Roy Company, Philadelphia, Pa.). In addition, some of the membranes were tested in a stirred batch reactor to study the effects of specific process variables such as tanning time. In these cases, 1–2g of collagen-papain membrane cut into small chips were used in a reaction volume of 25–50 ml. All experiments with beer were performed at the Research Laboratories of a brewery.

When gelatin was used as the substrate, the time course of the hydrolysis reaction was followed by measuring the decrease in viscosity of gelatin solution (Haurowitz, 1963). An Ostwald Viscometer was used for this purpose. Since gelatin forms a gel below 37°C, its viscosity cannot be measured at lower temperatures. Therefore, all assays were carried out at 43°C. In the concentration range of 0–5%, a linear relationship was obtained between gelatin concentration and relative viscosity of gelatin; i.e., the ratio of the viscosity of gelatin to that of water at the same temperature.

The ester hydrolysis of benzoyl arginine ethyl ester (BAEE) was followed by estimating the amount of base (0.1N sodium hydroxide) consumed while a constant pH was maintained during the reaction (Smith and Parker, 1958). In all cases, 25 millimolar BAEE containing 5 mmoles/liter of cysteine and 2 mmoles/liter of ethylene diamine tetraacetic acid (EDTA) was used as the substrate.

In a recycle reactor, the substrate spends only a fraction of the total time of experiment in the reactor. Therefore the observed initial reaction rates, based on the concentration history in the reservoir, were converted to true catalytic activities by multiplying by a correction factor. Detailed kinetic analysis revealed that collagen-papain recycle reactor system behaved like a perfectly mixed system (Venkatasubramanian and Shyam, 1974). Under these conditions, the true reaction rate (volume basis) is given by (Constantinides et al., 1973):

$$r_t = r_{obs} \cdot \frac{\tau_{Res} + \tau_R}{\tau_R} \qquad (1)$$

where r_t = true reaction rate; r_{obs} = observed reaction rate; τ_{Res} = residence time in the reservoir, and τ_R = residence time in the reactor.

Determination of the amount of enzyme bound to collagen. The proteinaceous nature of the carrier material, collagen, renders most of the common methods of protein determination inapplicable. Therefore, a suitable method had to be devised which would distinguish between the enzyme protein and the carrier protein. This was accomplished by taking advantage of the fact that papain contains tryptophan, while collagen does not. The method of Blackburn (1968) for tryptophan determination was suitably modified to estimate the tryptophan content of collagen-papain membranes.

A sample of the membrane weighing 20–30 mg (dry weight) was dissolved in 1 ml of 1N sodium hydroxide. To this, 9 ml of 21.4N sulphuric acid containing 30 mg of p-dimethylamino benzaldehyde was slowly added and mixed, while cooling in an ice bath. The mixture was stored in the dark for 1 hr. 0.1 ml of freshly prepared 0.04% sodium nitrite was then added to develop a blue color. After waiting for another 30 min, the absorbance of the mixture was read at 600 nm using a Beckman-DBG Spectrophotometer. The absorbance readings were obtained against a blank prepared in the same manner as above, but omitting the p-dimethylaminobenzaldehyde reagent. A linear relationship was found between absorbance readings and different papain concentrations. Blank collagen membranes without any enzyme on them, did not give any detectable background absorbance reading.

Chill-proofing of beer by the collagen-papain biocatalytic reactor. Actual chill-haze tests using a collagen-papain biocatalytic reactor were conducted at the Research Laboratories of a brewery. A collagen-papain module containing 15g of complex was used for all the tests. The membrane was prepared from the low specific activity papain preparation; the total amount of enzyme used was 1g, with a total activity of 500 IU. Three different test runs were performed. In all cases, primary filtered beer (without any additives) was passed continuously through the module at desired flow rates. To the product stream, foam stabilizer was added, followed by a final filtration and packaging. Chill-haze tests were conducted at the end of the desired periods of storage. Clarity of the processed beer was evaluated both visually and by means of a Coleman #9 nephelometer. These tests were performed according to the methods recommended by the American Society of Brewing Chemists. The nephelometer readings were correlated with visual observations in the order of decreasing clarity as outlined in Table 6.

RESULTS & DISCUSSION

THE AMOUNT of papain attached to collagen membranes by the methods of covalent binding and complexation are shown in Table 1. The catalytic activities of these membranes are also shown in this table. The expressed activity of covalently bound papain is 4.5-fold lower than that of a tanned membrane prepared

Table 1—Comparison of collagen-papain membranes prepared by covalent binding and by complexation

Binding method	Enzyme loading (g papain/ g immobilized derivative)	Expressed activity[a] (IU/g membrane)	Apparent sp. activity (IU/mg enzyme)	Specific activity of free enzyme (IU/mg)
Covalent attachment	0.075	137	1.8	12.7
Complexation, (untanned)	0.290	1100	3.8	12.7
Complexation, (tanned)	0.298	597	2.0	12.7
Complexation, (untanned)	0.131	30	0.229	0.5
Complexation, (tanned)	0.131	20	0.153	0.5

[a] In all cases, 25 mmoles/liter benzoyl arginine ethyl ester was used as the substrate. Tanning was done with 10% glutaraldehyde for 30 sec.

Table 2—Effect of tanning time on the activity of collagen-papain membranes

Tanning time (min)	Proteolytic activity[a] (mg gelatin hydrolyzed/min/g complex)
0	18.0
0.5	12.1
1.0	8.5
2.0	6.7

[a] Proteolytic activity of collagen-papain membranes were determined with 2% gelatin as substrate. 10% (w/v) glutaraldehyde at pH 6.0 (pH adjusted with sodium bicarbonate) was used as the tanning agent in all cases.

by the complexation process (column 3 of Table 1). The activity of untanned membrane is even higher. Based on the amount of enzyme actually immobilized as determined by the tryptophan analysis procedure, the apparent specific activities have been calculated. Again, these values are higher for the membranes prepared by the complexation procedure. It should also be noted that the amount of enzyme immobilized is in itself not of intrinsic importance; the extent of expressed catalytic activity is of greater practical value. From this point of view, collagen-papain membranes prepared by complexation are far superior to those prepared by covalent coupling. Therefore, all other data reported here were obtained from complexed collagen-papain membranes.

These results clearly demonstrate the superiority of the complexation process over the covalent binding procedure. In the latter procedure, papain is covalently conjugated to a *modified* collagen; i.e., one which has been chemically pretreated to permit covalent attachment of enzyme to it. The linkages are randomized and occur only wherever the carrier protein has been successfully chemically modified in such a process. Moreover, the nonspecific nature of covalent attachment of the enzyme can result in damage to some of the enzyme molecules. In contrast, the complexation procedure utilizes *unmodified* protein-protein interactions. It involves localization of the enzyme in specific domains of the collagen microstructure with the cooperative action of multiple bonds such as salt linkages, hydrogen bonding and van der Waal's interactions (Venkatasubramanian et al., 1974a). This leads to the formation of a stable network of collagen and the enzyme. Thus, it provides the nearest possible approach to the micro-environment which enzymes experience in cells, their natural habitat. Because of this it is possible to immobilize larger amounts of active enzyme per unit weight of carrier than other methods of enzyme immobilization. Furthermore, the enzyme bound in this way is highly stabilized.

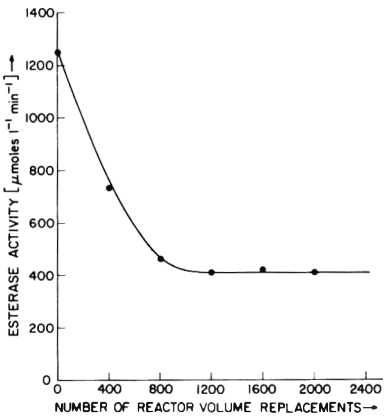

Fig. 1—Stability of a collagen-papain biocatalytic module on repeated contacts in a recycle reactor system. Assay conditions: 30°C, pH 7.0, recycle rate 400 ml/min. 25 mmoles/liter BAEE was used as substrate. The module contained 18g of collagen-papain complex.

It was also found that covalently coupled collagen-papain adducts were not structurally stable. After a few repeated contacts with the substrate, the material started disintegrating into small fibrils. The structure of untanned collagen-papain complexes was also relatively weak; nevertheless, it was much better than the covalently bound material.

Tanning with glutaraldehyde solution at an appropriate pH was found to greatly strengthen collagen-papain complex membranes. However, glutaraldehyde tanning also denatures some of the enzyme as seen in Table 1. 45% and 33% reduction in expressed activities is observed when the specific activities of the starting (free) enzyme were 12.7 and 0.5 IU/mg protein, respectively. The effect of tanning time on the activity of several membranes is shown in Table 2. As tanning time increases, structurally superior membranes are obtained with a concomitant decrease in the catalytic activity. A tanning time of 0.5 min was found to be a practical compromise. Further work is now in progress to determine the optimal combination of glutaraldehyde concentration and the treatment time of tanning.

The esterase activity of collagen-papain complex is shown in Figure 1, as a function of the number of reactor volume replacements. The initial decrease in activity is due to the desorption of the loosely bound enzyme. The stable limit of activity is found to be 410 μmoles/liter min. This corresponds to about 19% active enzyme by weight; i.e., the membrane exhibited an activity equal to that amount of soluble active enzyme which when immobilized in the membrane would constitute 19% of its total mass. A small portion of this membrane, when chipped and tested in a batch reactor, showed an activity corresponding to 7.2% (by weight) only. This clearly demonstrated the superior contact achieved in a spirally wound biocatalytic module, where the membrane is formed into an organized structural part of the reaction system.

The stability of collagen-papain complexes on repeated contacts, when gelatin was used as the substrate, is outlined in Table 3. The activity of the first run is somewhat lower. This may be because of the lack of establishment of an initial swelling equilibrium. On subsequent contact, there was a decrease in activity before a stable limit was reached. That this decrease was due to the elution of enzyme and not due to its inactivation was demonstrated in the following manner. A portion of the final reaction sample from Run #2 was incubated with a protein substrate, Azocoll® (Caliochem, La Jolla, Calif.). The amount of protein hydrolyzed roughly corresponded to the amount of eluted enzyme. Considering the macromolecular nature of the substrate gelatin, this result is not surprising. There are several documentations in the literature in which a similar phenomenon —even with covalently bound enzymes— has been reported (Epton et al., 1971; Silman and Katchalski, 1966). Once a stable limit was reached, there was not any significant further desorption of the

Table 3—Proteolytic activity of collagen-papain complex on repeated contacts in a batch recycle reactor

Run no.	Proteolytic activity[a] (g gelatin hydrolyzed hr^{-1} g $complex^{-1}$)
1	0.64
2	1.32
3	1.15
4	0.96
5	0.80
6	0.84
7	0.82

[a] In all cases, 2% gelatin was used as the substrate.

Table 4—Results of chill-haze tests—Run No. 1

Amt of beer processed (liters)	Pressure differential (psi)	Time of run	Flow rate (ml/min)	Chill-haze tests		
				Initial	1 month	3 months
Control beer with chill-proofing				Brill	P. brill	P. brill
13	15	21 min	605	Brill	Sl. veil	P. brill
13	10	38 min	342	Brill	P. clear	P. clear
13	5	6 hr 18 min	34.4	Brill	P. brill	P. clear
12	2.5	23 hr	8.7	Brill	P. brill	P. brill

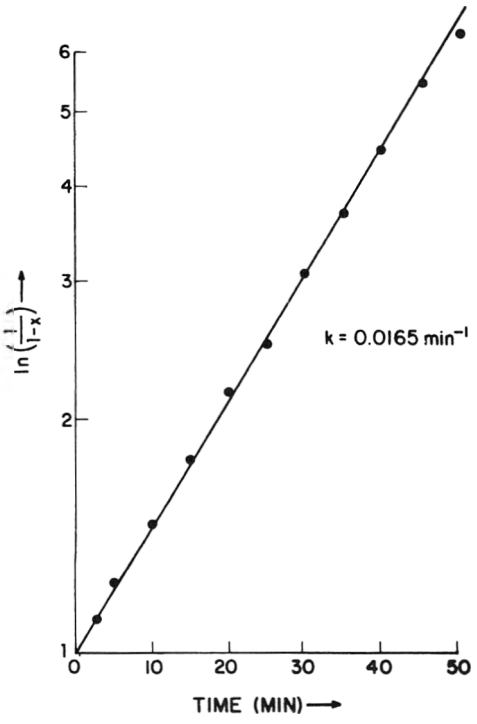

Fig. 2—Pseudo-first order plot of the esterolysis of BAEE catalyzed by collagen-papain module.

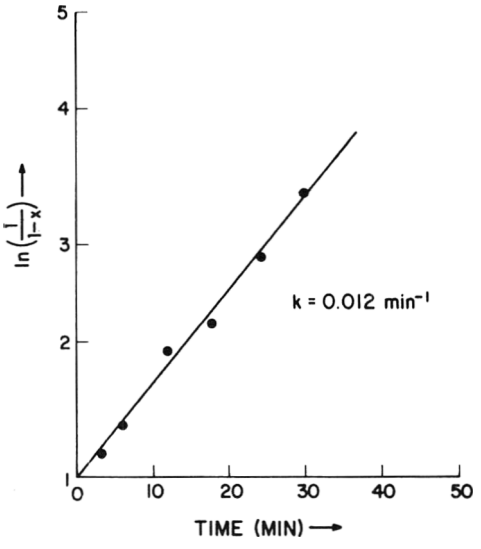

Fig. 3—Pseudo-first order plot of the hydrolysis of gelatin effected by collagen-papain module.

enzyme from the collagen matrix. However, there was a slow inactivation of the enzyme itself.

After 5 months of extensive continuous and intermittent usage in a brewery in the process of chill-proofing beer, the module retained 56% of its original stable limit of activity. Assuming that the inactivation of enzyme is a first order process, the half-life of the module could be estimated as 180 days.

In Figure 2 and 3, kinetic data on the esterolysis and the proteolysis reactions effected by immobilized papain are shown. These reactions could be represented as pseudo first order reactions. The first order kinetic constants are 0.0165 min^{-1} and 0.012 min^{-1}, respectively. The rate constant for the high molecular weight substrate is lower indicating possible diffusional resistances to the transport of the substrate molecules to the reaction site. Furthermore, with gelatin substrate, most of the catalytic action would be due to the enzyme molecules immobilized on or near the matrix surface. The smaller molecular weight BAEE can more readily penetrate into the carrier, thus becoming exposed to a larger number of enzyme molecules.

Three different chill-haze tests were performed. In Table 4, the results of the first test are shown. At the end of a 3-month chill-haze test, all samples were of acceptable quality. The effect of varying flow rates through the module is also seen from Table 4. At higher flow rates, the residence time available for the contact of the substrate is lower, with a consequently lower degree of protein hydrolysis. After the first run, two barrels of primary filtered beer were run through the module at a rate of 8–9 ml/min. This required about 20 days of continuous running. No samples were taken during this run. After this run, the first run was repeated, passing 13-liter samples through the module at different flow rates to find out if the module was still active. Table 5 shows the results of this run at the end of a 1-month chill-haze test. The module had apparently lost some of the enzymatic activity during this period. As indicated earlier, the module was reassayed in our laboratory with gelatin substrate and it was found to have lost 44% of its original activity. All the samples from both the runs were normal with respect to taste, color and foam stability.

Since prior to its use in chill-proofing, the module had reached a stable limit of activity (having lost the loosely bound enzyme through leaching) the decrease in enzymatic activity during the 5-month's period of use is more likely to have resulted from enzyme inactivation rather than its desorption from the collagen matrix. Our experience with other collagen-enzyme systems indicates that once a stable activity limit is reached by the immobilized preparation, further decline in activity with continued usage is essentially due to enzyme denaturation (Gilmore, 1974). Therefore, no enzyme leached from the matrix could possibly be present in the chill-proofed final product, thus meeting the future labelling requirements. However, more experimental work is needed to establish unequivocally whether the loss in observed catalytic activity is due to enzyme inactivation or enzyme desorption from collagen membrane.

These results demonstrate the technical feasibility of using a collagen-papain biocatalytic reactor for chill-proofing beer. The papain preparation used for this reactor had a low specific activity (0.5 IU/mg protein). By starting with a purer preparation and also by increasing the loading of the reactor, the performance of the reactor could be greatly improved.

Table 5—Results of chill-haze tests—Run No. 3

Amt. of beer processed (liters)	Pressure differential (psi)	Time of run	Flow rate ml/min	Chill-haze tests		
				Initial	1 month	3 months
Control beer with chillproof				Brill	P. brill	P. brill
13	12	20 min	650	Brill	P. clear	Sl. veil
13	8	36 min	342	Brill	V.Sl. veil	Veil
13	3.5	6 hr	36.1	Brill	P. clear	Veil
13	1.25	23 hr	8.7	Brill	V. Sl. veil	Sl. veil

Table 6—chill-haze tests—Correlation of nephelometer readings with visual observations of beer clarity[a]

Nephelometer reading	Visual observation
9–12	Brilliant
12–14	Practically brilliant
14–16	Clear
17–18	Practically clear
19–22	Very slight veil
23–27	Slight veil
> 28	Veil

[a] Grades down to p. clear are acceptable as properly treated beer.

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UTILIZATION OF OLIGOSACCHARIDES BY LACTIC ACID BACTERIA DURING FERMENTATION OF SOY MILK

INTRODUCTION

SOYBEANS are a rich source of protein; yet, predominantly soybean-based foods are not widely accepted mainly because of their beany flavor and secondarily because of the belief that they cause flatulence. Preparation of fermented foods using lactic acid bacteria has been suggested to improve their acceptability (Hang and Jackson 1967; Matsouka et al., 1968). Angeles and Marth (1971) reported that soy milk serves as an excellent medium for the growth of lactic acid bacteria. Sufficient acid production is a prerequisite for the manufacture of fermented products and depends upon the ability of the organism to utilize the available carbohydrates in the medium. The fermentable carbohydrates in soybeans and soybean products are low molecular weight oligosaccharides such as sucrose, raffinose, and stachyose (Kawamura, 1967; Shallenberger et al., 1967). Stachyose, a tetrasaccharide, is made up of three simple sugars fructose, glucose, galactose (Fig. 1). These sugars are joined in such a manner that the stachyose molecule may be considered to contain the nonreducing sugar moieties sucrose and raffinose and reducing sugar moieties galactobiose, melibiose and manninotriose. When the galactosido-sucrose series of sugars are ingested, two initial enzymes are required to completely hydrolyze these oligosaccharides. An invertase (EC 3.2.1.26) is required to hydrolyze the sucrose moiety of the oligosaccharides and an α-galactosidase (EC 3.2.1.22) is necessary to hydrolyze the remainder of the molecule. Since the human gastrointestinal tract does not possess α-galactosidase (Gitzelmann and Auricchio, 1965), the metabolic fate of soybean galacto-oligosaccharides is uncertain. It has been suggested that raffinose and stachyose are primarily responsible for flatulence often experienced by persons consuming soybean foods. Richards and Steggerda (1966) using dogs, found that intestinal gas production could be related to the intake of soybean meal. Steggerda and Dimmick (1966) studied the effect of bean diets and concluded that flatus appeared to be related to chemical composition rather than fiber content of the product. Using human subjects, Steggerda et al. (1966) reported that gas-producing factor resides mainly in the low molecular weight oligosaccharide fraction of the soybean. Several other investigators have also suggested that raffinose and stachyose may be involved in flatulence resulting from ingestion of soybeans (Rackis et al., 1967; Murphy, 1969; Rackis et al., 1970). However, Rockland et al. (1969) concluded that flatulence factor in soybeans is other than these simple sugars.

A number of biochemical changes are known to occur during fermentation of soybeans. However, very little information is available on the changes in soybean oligosaccharides during fermentation or other processes. Shallenberger et al. (1967) reported a marked decrease in stachyose content during tempeh fermentation. Sugimoto and Van Buren (1970) showed that small amounts of partially purified cell extracts from *Aspergillus saitoi* possessing both invertase and α-galactosidase, yet free of protease activity added to soy milk resulted in complete hydrolysis of galacto-oligosaccharides. East et al. (1972) and Hsu et al. (1973) reported that raffinose and stachyose disappeared as a result of germination of soybeans.

Lactic acid bacteria possessing the ability to utilize sucrose, the major sugar found in soybeans, can be successfully used to manufacture fermented products from soy milk (Mital et al., 1974). Some lactic acid organisms also possess α-galactosidase and may thus utilize galacto-oligosaccharides such as raffinose and stachyose for growth and acid production (Mital et al., 1973). The purpose of this investigation was to determine the extent of utilization of soybean oligosaccharides by lactic acid bacteria during fermentation of soy milk.

MATERIALS & METHODS

Cultures

Lactobacillus cellobiosis (NRRL-B-1840) and *L. fermenti* (NRRL-B-585) were kindly supplied by Dr. William C. Haynes (USDA Northern Regional Research Lab., Peoria, Ill.). *Lactobacillus plantarum* (B-246) was given to us by Dr. J.R. Stamer (NYS Agr. Exp. Station, Geneva, N.Y.). *Streptococcus thermophilus* (Marshall) obtained from Dr. H.B. Naylor (Dept. of Microbiology, Cornell University, Ithaca, N.Y.) was also included in this study. The cultures were maintained by biweekly transfer in sterile litmus milk or soy milk and held at 5°C between transfers. Lactic broth

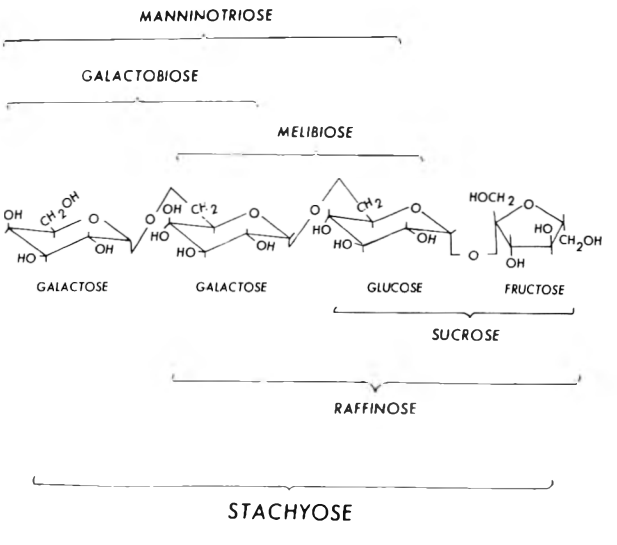

Fig. 1—Structure of stachyose.

Table 1–Relative responses of TMS derivatives of carbohydrates

Carbohydrate	Molecular wt	Silyl groups	Optimum temp (°C)	Retention time (min)
Mannitol	182	6	204	5.34
Inositol	180	6	216	6.60
Sucrose	342	8	267	11.70
Raffinose	504	11	>300	19.20
Stachyose	666	14	>300	33.80

Table 2–Recovery of added stachyose from soy milk

Stachyose added (%)	Total stachyose calculated (%)	Total stachyose found (%)	Recovery (%)
0.00	–	0.39	–
0.23	0.62	0.59	95
0.33	0.72	0.71	98
0.50	0.89	0.84	94
0.60	0.99	1.02	103

(Elliker et al., 1956) was used for propagation of cultures in liquid medium.

All the cultures were transferred in soy milk daily for 3 days before they were used to prepare 16–18 hr inocula used in these experiments.

Preparation of soy milk

Dry, whole soybeans (variety Harasoy) were thoroughly washed and soaked in water at 60°C until the absorbed water was about 1 ml/g dry weight. The soak water was decanted and the beans were washed. The beans were ground in a Waring Blendor for 5 min (3 min at low, 1 min at medium and 1 min at high speed) with boiling water. The ratio of beans to water was 1:9 (w/v) and the temperature of the water during grinding between 85–95°C. The resulting suspension was filtered under vacuum using a Büchner funnel equipped with a 7-in. standard Agway GLF milk filter with one layer of coarse pad on top of a fine pad. The resultant soy milk was dispensed in 160 ml screw-cap bottles, autoclaved for 15 min at 121°C and held at 5°C until further use. Typical soy milk composition was as follows: protein (N × 5.71)–3.31%; fat–2.20%; ash–0.49%; moisture–91.77%; carbohydrate (by difference)–2.23%.

Growth and changes in pH

100 ml of soy milk were brought to the temperature of incubation and inoculated with 1 ml of 16–18 hr test culture as described earlier. The inoculated medium was incubated at 30°C for *L. plantarum* and at 37°C for other organisms. Samples were withdrawn at selected intervals and analyzed for growth, residual sugars and changes in pH.

Growth of the organisms in soy milk was determined by plate counts using lactic agar (Elliker et al., 1956). Duplicate plates were incubated for 48 hr at 30°C for *L. plantarum* and at 37°C for other organisms. Changes in pH were followed using Beckman Zeromatic pH meter.

Sucrose broth

Lactic broth (Elliker et al., 1956) containing 1% sucrose as the only energy source was used to study the fermentation of sucrose by *L. cellobiosis.*

Analysis

Varian Aerograph gas chromatograph model 204, equipped with flame ionization detector was used for quantitative determination of silylated derivatives. The column consisted of 3 ft stainless steel tubing, 1/8 in. o.d., packed with 3% OV-1 on Chromosorb W (HP) 80–100 mesh (Pierce Chemical Co., Rockford, Ill.). The column was conditioned at 320°C and conditioned again using Silyl-8 (Pierce Chemical Co., Rockford, Ill.) at 200°C before analyzing the samples. The carrier gas was nitrogen with flow rate 30 ml/min and the injector temperature was 268°C. The oven temperature was programmed from 150–300°C at 10°C/min and retained at 300°C until the stachyose peak appeared. Hydrogen flow rate was adjusted to give maximum performance. Use of high temperature septums (Applied Science Lab. Inc., State College, Pa.) eliminated the problem of septum bleed at high temperatures. The sample volume injected was 5 μl.

Internal standard for gas chromatography

Inositol (Eastman Kodak Co., Rochester, N.Y.) was used as the internal standard for determining quantitative changes in soybean oligosaccharides during fermentation. Mature, dry soy beans contain inositol in the range of 1.9–2.6 mg/g as a component of phosphatidyl inositol and phytin (Liener, 1972). This is considerably diluted during preparation of soy milk. Preliminary investigations showed that no cleaving of inositol from phosphatidyl inositol or phytin occurred during the experimental conditions nor did the organisms used in this investigation ferment inositol. The analysis of unfermented soy milk revealed trace amounts of inositol which remained unchanged throughout the fermentation period.

Preparation of trimethylsilyl (TMS) derivatives

1 ml of the sample was added slowly with agitation to 2.0 ml of 1.8% barium hydroxide solution followed by 2.0 ml of 2.0% zinc sulfate solution. After allowing to stand for 10 min, the mixture was transferred to a 15-ml graduated, clean, dry centrifuge tube and centrifuged in an International Clinical centrifuge model CL no. 8562 E (International Equipment Co., Boston, Mass.) until a clear supernatant was obtained (usually 5 min). 1 ml of the supernatant was transferred to an 8-ml screw-cap vial with teflon liner. 1 ml of the internal standard (inositol 0.2 mg/ml aqueous solution) was added. The mixture was frozen at –40°C and lyophilized to complete dryness in a Virtis freeze dryer (Virtis Co. Inc., Gardiner, N.Y.). The procedure developed by Sweeley et al. (1963) was

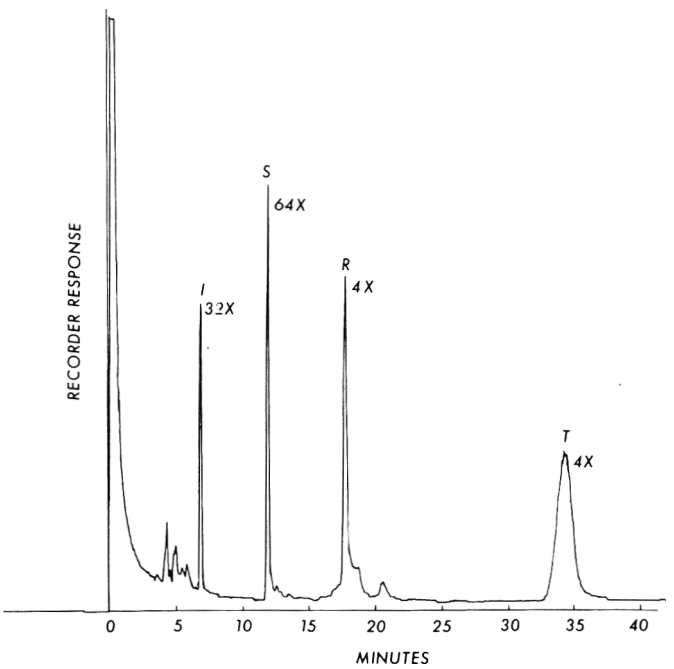

Fig. 2–Gas chromatogram of unfermented soy milk. Peak identities are: I, Inositol; S, Sucrose; R. Raffinose; T, Stachyose.

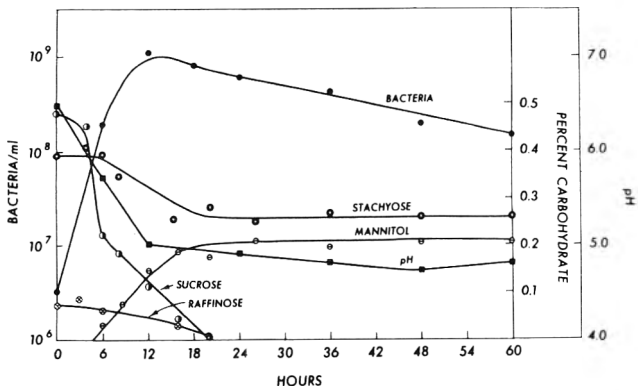

Fig. 3–Changes in bacterial numbers, pH and carbohydrate contents during fermentation of soy milk with L. cellobiosis.

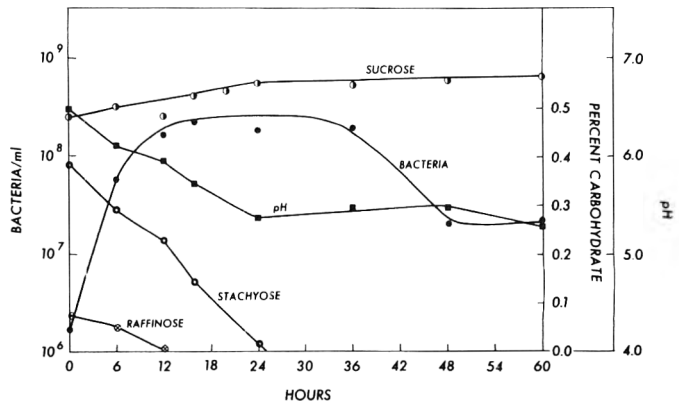

Fig. 5–Changes in bacterial numbers, pH and carbohydrate contents during fermentation of soy milk with L. fermenti.

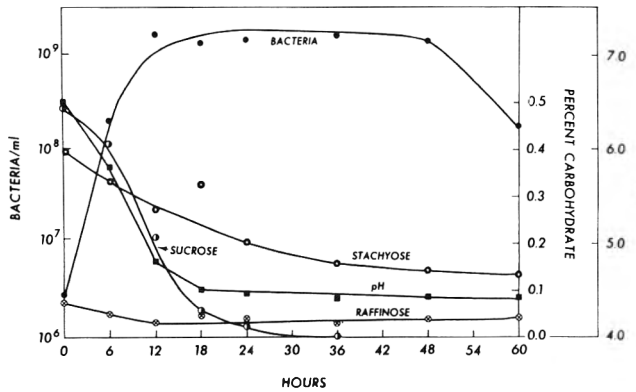

Fig. 4–Changes in bacterial numbers, pH and carbohydrate contents during fermentation of soy milk with L. plantarum.

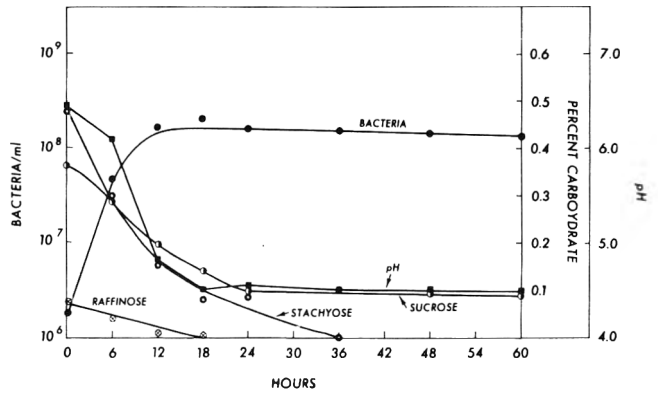

Fig. 6–Changes in bacterial numbers, pH and carbohydrate contents during fermentation of soy milk with mixed culture of L. fermenti and S. thermophilus.

used for preparation of TMS derivatives. 1 ml of Tri-Sil (Pierce Chemical Co., Rockford, Ill.) was added to the lyophilized sample and silylation was completed at 75°C for 1 hr.

The peaks were identified by comparing the retention times of TMS derivatives of pure sugars. The areas of peaks corresponding to mannitol, sucrose, raffinose and stachyose were calculated by triangulation as the product of height × width at half height. The ratio of the peak area obtained using anhydrous sugar to inositol was plotted against concentration of the sugar and was used for quantitative determination of the sugars in the sample.

The relative responses of TMS derivatives of carbohydrates are presented in Table 1. Each sugar eluted as a single symmetrical peak. The carbon contents in each of these carbohydrates varied after trimethylsilylation. Complete separation of mannitol and inositol with the same carbon contents but slightly different molecular weights was achieved with reasonable retention times. Retention times increased with increase in number of hydroxyl groups silylated and molecular weights. When known amounts of stachyose were added to soy milk, recoveries of 94–103% were obtained (Table 2).

RESULTS & DISCUSSION

SOY MILK (moisture 91.63%) contained 0.48% sucrose, 0.07% raffinose and 0.39% stachyose (Fig. 2). A few peaks appearing in the first 5 min indicated the presence of trace amounts of compounds in the soy milk with retention times similar to glucose, galactose and fructose. Since these monosaccharides were present only in trace amounts, no further attempt was made to identify them. Kawamura and Tada (1967) and Sugimoto and Van Buren (1970) also found trace amounts of monosaccharides in soybeans and soybean products. However, East et al. (1972) did not detect monosaccharides in dry, mature soybeans. The changes in oligosaccharide contents, viable counts and pH during fermentation of soy milk with different lactic acid bacteria are presented in Figures 3–6. The utilization of oligosaccharides was related to the growth of the organisms in the medium during early stages of fermentation. The population of the organisms increased for 16–18 hr and declined thereafter. The content of different oligosaccharides also decreased significantly during this period.

Fermentation of soy milk with *L. cellobiosis* resulted in complete utilization of sucrose and raffinose within 20 hr (Fig. 3, 7). Stachyose concentration decreased 35% during this period and remained unchanged thereafter. Unfermented soy milk does not contain mannitol. However, it appeared after 6 hr of fermentation of soy milk with *L. cellobiosis* and increased in concentration until 20 hr, the time needed for complete utilization of sucrose.

Hydrolysis of sucrose gives rise to equimolar amounts of glucose and fructose. Homofermentative and heterofermentative lactic acid bacteria differ with respect to fermentation of fructose (Horecker, 1962). Heterofermentative species possess mannitol dehydrogenase (mannitol:NAD oxidoreductase) (EC 1.-

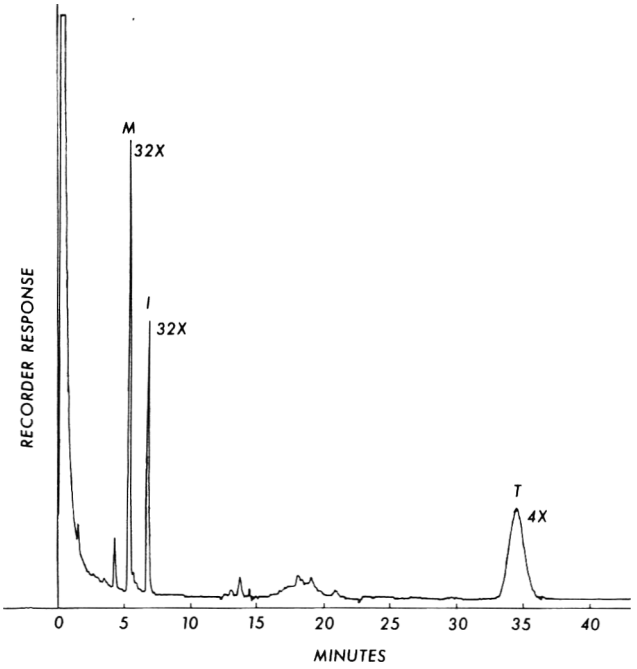

Fig. 7–Gas chromatogram of soy milk fermented with L. cellobiosis for 60 hr. Peak identities are: M, Mannitol; I, Inositol; T, Stachyose.

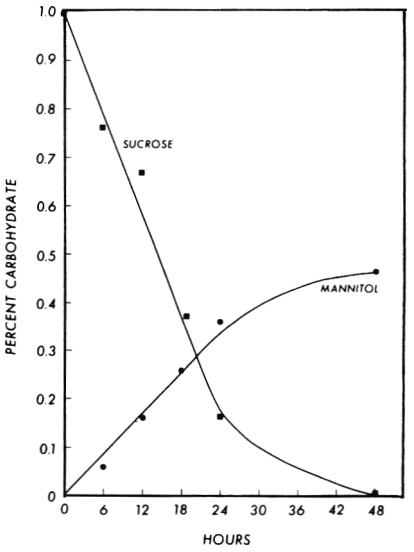

Fig. 8–Formation of mannitol in sucrose broth fermented with L. cellobiosis.

1.1.67) and can therefore reduce this ketohexose to the corresponding polyalcohol, mannitol:

$$\text{Fructose} + \text{NADA}^+ + \text{H}^+ \rightarrow \text{Mannitol} + \text{NAD}$$

L. cellobiosis, a heterofermentative lactobacillus, consequently reduced fructose to mannitol. The results obtained by fermenting sucrose broth with *L. cellobiosis* confirm that mannitol is an end product of sucrose fermentation (Fig. 8). 90–92% of fructose released in soy milk or broth medium as a result of sucrose hydrolysis was converted to mannitol. Persistence of mannitol peak both in soy milk and broth suggests that mannitol is not fermented by *L. cellobiosis*.

Fermentation of soy milk with *L. plantarum* showed that sucrose completely disappeared after 30 hr whereas raffinose content decreased slightly during early stages of fermentation and remained constant thereafter (Fig. 4). Only 60% of the stachyose was utilized. In the tempeh fermentation, Shallenberger et al. (1967) observed that stachyose content decreased markedly while sucrose decreased slightly. They also found that raffinose concentration remained unchanged throughout the fermentation because stachyose breakdown replenished raffinose as rapidly as it was hydrolyzed. A reducing disaccharide appeared between 35 and 60 hr and was identified as melibiose.

L. fermenti completely utilized raffinose and stachyose by 12 and 25 hr, respectively (Fig. 5). No mannitol was formed since sucrose was not fermented. Previous investigations (Rogosa and Sharpe, 1959; Sub-Committee of the International Comittee on Nomenclature of Bacteria of the International Association of Microbiological Societies, 1968) showed that some strains of *L. fermenti* utilize sucrose. However, the *L. fermenti* strain (NRRL-B-585) used in this study did not ferment sucrose. The slight increase observed in sucrose concentration was due to release of sucrose moiety from raffinose and stachyose hydrolysis. Fermentation of soy milk by mixed culture of *S. thermophilus* and *L. fermenti* showed complete disappearance of raffinose and stachyose whereas 0.08% of sucrose remained unfermented (Fig. 6). However, fermentation of soy milk with only *S. thermophilus* resulted in complete utilization of sucrose whereas raffinose and stachyose contents remained unchanged.

Delente and Ladenburg (1972) reported that treatment of soybean meal with a mixture of invertase and α-galactosidase resulted in quantitative conversion of oligosaccharides into monosaccharides. Melibiose and manninotriose appeared as intermediate products. Sugimoto and Van Buren (1970) showed that treatment of soy milk with an enzyme preparation from *Aspergillus saitoi* completely decomposed all the oligosaccharides to their constitutive monosaccharides. They also observed that melibiose and manninotriose appeared as intermediate products. Mital et al. (1973) also found that treatment of stachyose with an enzyme preparation from *L. cellobiosis* resulted in an increase in the concentrations of monosaccharides with time. Melibiose and manninotriose appeared as intermediate products but decreased in concentration with time. However, we did not detect reducing mono-, di-, or tri-saccharides during fermentation of soy milk with lactic acid bacteria. The monosaccharides formed as a result of hydrolysis of oligosaccharides were apparently utilized by the organisms for growth and reducing di- or tri-saccharides were further hydrolyzed.

L. cellobiosis and *L. fermenti* possess α-galactosidase (Mital et al., 1973) while induction of this enzyme by melibiose in *L. plantarum* has been demonstrated (Mital, 1974). However, no melibiose was detected in soy milk. It, therefore, appears that raffinose acts as an inducer in soy milk. Raffinose has been demonstrated to induce α-galactosidase in *E. coli* (Lester and Bonner, 1957; Sheinin and Crocker, 1961). The α-galactosidases from lactobacilli are active between pH 4.5 and 8.0 and exhibit pH optima in a rather narrow range (5.2–5.9) (Mital et al., 1973). α-Galactosidase activity of these organisms declined sharply as the pH of the medium reached 4.5 resulting in only partial utilization of stachyose by *L. cellobiosis* and *L. plantarum*.

The results of this investigation indicate that lactic acid bacteria possessing α-galactosidase utilize galacto-oligosaccharides present in soy milk. Thus, lactic fermentation can be used to reduce raffinose and stachyose contents which are believed to contribute to flatulent properties of soybeans. However, rapid utilization of sucrose resulted in a pH low enough to inhibit further utilization of higher saccharides and proved to be a limiting factor in their complete removal.

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Staphylococcus aureus GROWTH AND SURVIVAL IN MACARONI DOUGH AND THE PERSISTENCE OF ENTEROTOXINS IN THE DRIED PRODUCTS

INTRODUCTION

MACARONI and noodles are inexpensive and popular food items. Macaroni can be made solely from durum or regular wheat, but the U.S. standard for noodles requires the addition of 5.5% egg or egg yolk (CFR, 1973). Macaroni and noodle products may also be enriched with vitamins, minerals and soy flour to increase their nutritional values. These enriched foods are readily accepted by the public. Macaroni and noodles are produced by mixing tap water, semolina, farina, flour and other ingredients to form a stiff dough with about 30% moisture. The dough is extruded or rolled into a variety of shapes and forms and then dried to 10–12% moisture at about 40°C. Drying must be done slowly and precisely or the products may crack under stress (Hoskins, 1970). It should be noted that the macaroni and noodle products are not subjected to cooking or heating during manufacturing and the drying temperatures allow the rapid multiplication of bacteria. Recent foreign publications reported the presence of 10^3–10^6 of *S. aureus* per gram in pasta products (Matejovska et al., 1972; Vamos, 1969). Routine surveillance by the Food & Drug Administration showed *S. aureus* contamination of some pasta products manufactured in this country. Concern over the potential microbiological hazards of these products prompted the research reported here. Our primary aim was to determine the potential for growth and enterotoxin formation by *S. aureus* in pasta dough as well as their persistence in the dried finished products. The results are discussed in relation to current pasta manufacturing practices.

EXPERIMENTAL

Culture and media

S. aureus strain 743 which produced enterotoxin A, *S. aureus* strain S6, which produced enterotoxins A and B, and *S. aureus* strain 137, which produced enterotoxin C, were inoculated in wet dough to test for enterotoxins A, B and C formation. The inocula of these strains were grown in brain heart infusion broth overnight at 35°C. Appropriate dilutions in tap water were added to semolina to make the dough. Bacteriological examinations of samples were tested according to the procedures of the current edition of the FDA *Bacteriological Analytical Manual for Foods* (FDA, 1972).

Procedure for preparing inoculated dough and noodle

Inoculated dough and noodles were made by blending 2.5 kg of semolina with 500 ml of a diluted suspension of *S. aureus* in tap water. In the noodle mix, 130g of dried egg yolk was added into the tap water mix. Blending was achieved by mixing first in a Hobart mixer and then in a twin-shell dry blender. The above mixture was friable and could be blended into a uniform mixture. After blending, the mixture was returned to the Hobart mixer and 200–300 ml more water was added to make a stiff dough. The dough was formed into a 500g ball or rolled into noodle-shaped products. Dough and noodle samples were incubated in partially open polyethylene bags at 25° and 35°C for various periods up to 30 hr. Noodle-shaped products were dried on a rack with forced-air circulation inside the chamber of a New Brunswick G 25 incubator. The drying temperature was controlled at 40°C but the humidity was not as strictly controlled as it would be under commercial conditions. However, the drying chamber served the purpose for these bacteriological studies.

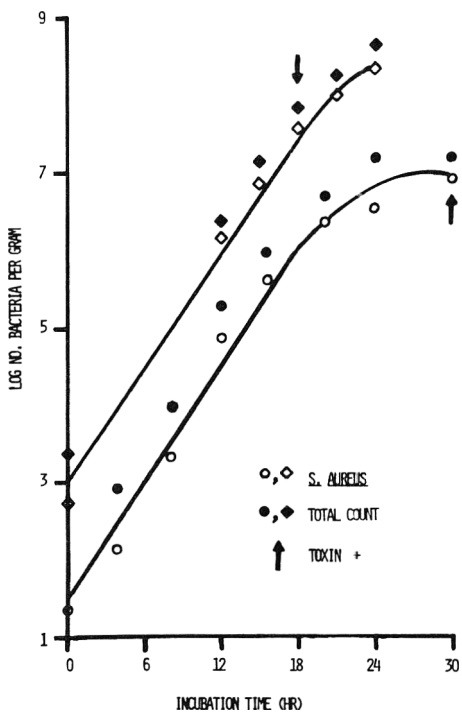

Fig. 1–Growth of S. aureus and enterotoxin A formation in pasta dough with two inoculum levels at 25° C.

Qualitative enterotoxin and nuclease analysis

Pasta was stored frozen and tested for enterotoxins by the method of Casman and Bennett (1965). Heat stable staphylococcal nuclease was analyzed by the method of Lachica et al. (1972).

RESULTS & DISCUSSION

Growth of S. aureus and formation of enterotoxins

Initial inoculation studies indicated that *S. aureus* grew well in wet dough made with semolina and tap water. Its growth rate and enterotoxin formation were not affected by the addition of 5.5% egg yolk, nor by the physical shape of the dough (i.e., lump or noodle-shaped). The growth rate and the time of enterotoxin A formation of *S. aureus* strain 743 at two inoculum levels at 25°C are presented in Figure 1. The semolina had about 2,500 aerobic bacteria per gram and had no *S. aureus* contamination. Enterotoxin A was detected after 30 hr of incubation in the sample with approximately 20 of *S. aureus* inoculum per gram, but it was detected after 18 hr of incubation with an initial inoculum of approximately 600 of *S. aureus* per gram. At 35°C, enterotoxin A formation was more rapid than at 25°C (Fig. 2). The growth of *S. aureus* in the dough was also better and a higher population level was reached than at 25°C (Fig. 1 and 2). The generation time of *S. aureus* strain 743 in pasta dough was about 50 min at 35°C and 70 min at 25°C. Enterotoxin was detected many times after the time indicated by the arrows in Figures 1 and 2 but it was never detected before the indicated time. The minimum *S. aureus* population for enterotoxin development in the dough was between 7×10^6 to 3×10^8 cells per gram.

Other *S. aureus* strains also grew well in wet dough made with only semolina and water. *S. aureus* S6 strain normally produced 100-fold more enterotoxin B than A in suitable media or foods (Genigeorgis et al., 1969). *S. aureus* strain S6 grew well in the wet dough but produced only enterotoxin A without any detectable trace of enterotoxin B. *S aureus* strain 137 also grew well in wet dough and produced enterotoxin C.

Heat-stable nuclease is a distinctive

characteristic of *S. aureus* and has been proposed by Lachica et al. (1972) as an indicator of gross ($> 10^7$/g) *S. aureus* contamination in foods. Surprisingly, no nuclease activity could be detected from the toxic dough samples prepared with all three strains of *S. aureus*. It appeared that enterotoxin B and nuclease formation were restricted in the pasta dough.

Persistence of S. aureus and the enterotoxins in storage and cooking

To test the stability of *S. aureus* and its enterotoxins in the dried products, inoculated doughs were shaped into noodles which were incubated for 24 hr at 35°C and then dried to contain about 11% moisture at 40°C. Table 1 shows that the *S. aureus* cells were nonviable after 90 or 180 days of storage at 25°C. In contrast, enterotoxin A was stable and could be recovered after 1 yr (Table 1). Enterotoxin C was also detected in one dry sample after more than a year of storage. For comparison, *Salmonella typhimurium* strain FDA 900 was blended into noodle-shaped products and dried at 40°C. Unlike *S. aureus*, *S. typhimurium* was recovered from the dried products after 1 yr of storage (Table 1). Therefore, macaroni and noodle samples should be examined for *S. aureus* contamination within 30 days of manufacturing because of the rapid die-off rate of this bacterium. Even so, heavily contaminated macaroni and noodle samples may be toxic in the absence of viable *S. aureus* following aging.

Staphylococcal enterotoxins were reported to be heat-stable and survived more than 1 hr of boiling at 100°C (Denny et al., 1966; Fung et al., 1973). Macaroni and noodles are normally cooked in boiling water and rinsed. The stability of enterotoxins A and C in noodles following cooking was tested. Toxic dried noodles were boiled in 10 volumes of 0.85% saline for 10 min, drained, and then rinsed with 10 volumes of cold saline. The saline was discarded and the cooked samples were analyzed for toxins. Enterotoxin was recovered in one out of three trials. It appeared that normal cooking would rinse out part of the staphylococcal enterotoxins from the cooked pasta.

We have shown that staphylococcal enterotoxins can be formed in wet warm dough after overnight incubation. The possibility of this occurring during usual conditions of manufacture is fairly remote because the short period of time involved during initial drying to 16–18% moisture level (a_w 0.90) inhibits the growth of *S. aureus* (Troller, 1972). *S. aureus* growth is usually limited to 3–6 generations in the commercial drying process (Walsh, 1973). Generally, it has been our experience that the total number of *S. aureus* found in fresh commercial samples was much below the population level of *S. aureus* associated with toxin production. Thus, the hazard of staphylococcal enterotoxins formation in domestic pasta would seem to be fairly remote; nevertheless, the hazard potential remains. Our experience with the industry in connection with FDA's Cooperative Quality Assurance Program indicates that macaroni and noodles can be manufactured free of *S. aureus* with modern equipment and under good manufacturing practices such as those advocated by Winston (1972). This position is reflected in a microbial guideline issued by the National Macaroni Association, which recommended a negative tolerance for *S. aureus* (Winston, 1971).

Table 1—The persistence of bacteria and enterotoxin A in dried pasta stored at 25°C

Storage time (days)	Salmonella typhimurium (per gram)		Staphylococcus aureus (per gram)		Enterotoxin A	
	Semolina	Semolina +Yolk	Semolina	Semolina +Yolk	Semolina	Semolina +Yolk
0	23	9	10^9	8×10^8	+	+
30	NT[a]	NT[a]	2×10^7	NT	+	NT
90	1	2	10^3	0	+	+
180	1	9	0	NT	+	NT
360	1	9	0	0	+	+

[a] NT = Not tested

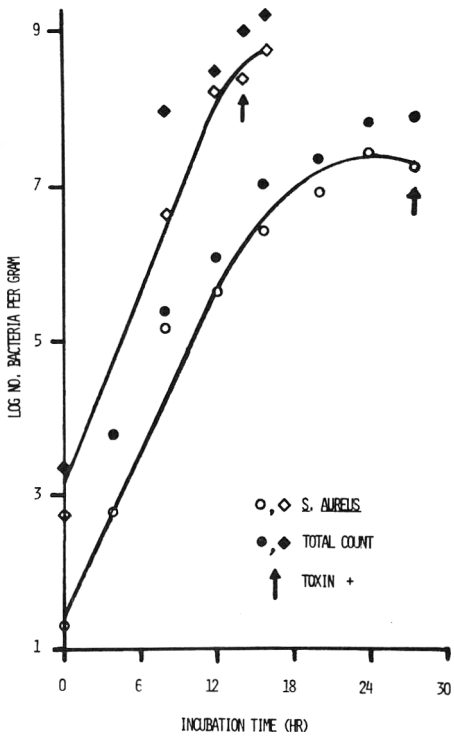

Fig. 2—Growth of S. aureus and enterotoxin A formation in pasta dough with two inoculum levels at 35° C.

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APPLIED SCIENCE and ENGINEERING

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DEVELOPMENT OF A CHEMICAL TEST FOR SHRIMP QUALITY

INTRODUCTION

FOR MANY YEARS investigators have sought to develop a simple and reliable chemical test for shrimp quality. Fieger and Friloux (1954) concluded that amino nitrogen (AA-N) appeared to have considerable merit as an index of quality for unspoiled shrimp while trimethylamine (TMN) content and volatile acid content were of no value. Bailey et al. (1956) indicated that glycogen-sugar, lactic acid and acid soluble orthophosphate contents could be used for "relative" comparison of shrimp during their prime quality phase. Other useful tests suggested by Bailey et al. were pH, amino nitrogen, degree of hydration of water-insoluble protein and B-vitamin content. Bethea and Ambrose (1961, 1962) proposed that pH and picric acid turbidity of shrimp and shrimp drip were useful indicators of quality. Gagnon and Fellers (1958) suggested that volatile base and the ratio of volatile base to total nitrogen were good indicators of quality of frozen breaded shrimp. Iyengar et al. (1960) suggested that total volatile nitrogen (TVN) and TMN content of shrimp were not good indicators of shrimp quality because of leaching due to the washing effect of melting ice. However, they suggested that pH and a catechol-ferric chloride test were useful indices of shrimp quality. Farber (1954) suggested that volatile reducing substances were a more effective index of shrimp quality than was TVN or TMN. Montgomery et al. (1970) have indicated that total volatile base (TVN) and TMN were useful indices of quality for Australian shrimp. The limit of acceptability in some sectors of the Australian marketing system and Japan has been established as 5 mg TMN and/or 30 mg TVN. A recent report by Flick and Lovell (1972) suggests that hypoxanthine content might be a good indicator of shrimp quality. Of the various chemical tests suggested, only pH appears to be used to any extent by the shrimp processing industry in the United States. In general it is not considered as a reliable index.

Recently, Cobb et al. (1973a) reported a high correlation between total volatile nitrogen/amino-nitrogen ratio (TVN/AA-N) and quality of shrimp. This report shows (1) the applicability of this test to potential shelf life and bacterial count estimations of shrimp and (2) factors affecting TVN analysis. The enzymic basis of the test is discussed.

MATERIALS & METHODS

WHITE SHRIMP (*Penaeus setiferus*) and brown shrimp (*Penaeus aztecus*) were obtained directly from waters of the Northwestern Gulf of Mexico and bordering bays and from commercial sources. Various species of frozen shrimp were obtained from commercial sources along the Gulf Coast. Shelf-life studies were conducted by placing shrimp in sterile ice as previously described (Cobb et al., 1973c). The period in sterile ice during which shrimp retained an acceptable odor was defined as the potential shelf life of the shrimp.

Microbiological and biochemical analyses

Total plate counts were performed as described by Vanderzant et al. (1970). Trichloroacetic acid extracts and chemical analyses were conducted as previously described (Cobb et al., 1973a). Amino nitrogen was determined by the copper procedure and/or by use of a fully automated Beckman Model 120 C Amino Acid Analyzer. TVN was determined by the microdiffusion procedure with $Na_3PO_4 \cdot KOH$ or saturated Na_3PO_4 as releasing agent.

Organoleptic analyses

Organoleptic evaluations were made on the basis of odor and appearance by trained personnel. Spoilage was assumed to be 30 mg volatile nitrogen/100g shrimp as indicated by Montgomery et al. (1970).

Calculations

All calculations were based on an 80% water content in the shrimp. Standard deviations, regression lines and correlation coefficients were calculated according to Sokal and Rohlf (1969).

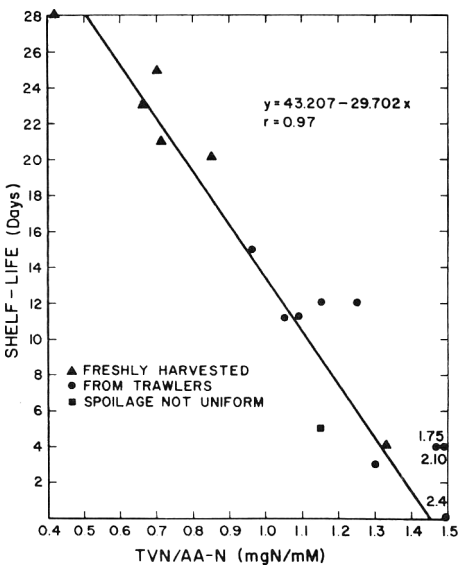

Fig. 1—Relationship between TVN/AA-N ratio and potential shelf life of brown shrimp.

RESULTS & DISCUSSION

Correlation of TVN/AA-N ratio with potential shelf life of brown shrimp

In Figure 1 is presented the relationship between the potential shelf life (PSL) and TVN/AA-N ratio in brown shrimp. Included are analyses from freshly harvested shrimp and shrimp taken from commercial trawlers. There is a sig-

Table 1—Extraneous volatile nitrogen (ammonia) produced by hydrolysis of asparagine and glutamine in shrimp extracts treated with 7N NaOH

Time (hr)	Ammonia (mM/100g)	Aspartic acid (mM/100g)	Glutamic acid (mM/100g)	Δ Ammonia (mM/100g)	Δ Aspartic acid and glutamic acid (mM/100g)
0	1.11	0.05	0.27	–	–
2	1.97	0.16	0.65	0.86	0.49[a]
3	1.98	0.17	0.65	0.85	0.50
4	2.09	0.24	0.77	0.98	0.69

[a] Increases in aspartic and glutamic acids indicate hydrolysis of asparagine and glutamine.

nificant ($P < 0.01$) negative correlation ($r = -0.97$) between the TVN/AA-N ratio and PSL with an extrapolated value of 1.45 mgN/mM as zero shelf life or spoilage. Some samples from commercial trawlers had been heavily treated with sodium bisulfite. The data from these samples and from one sample with non-uniform spoilage did not fit the curve well. Several samples, which had been treated with bisulfite and had high TVN/AA-N ratios, developed spoilage odors after 3–4 days ice storage. This may represent the period of time to lower the concentration of bisulfite sufficiently for spoilage odors to appear. The results from two bisulfite-treated samples were plotted on Figure 1 but were not included in the calculations.

Correlation of TVN/AA-N ratio with total plate counts of shrimp

The TVN/AA-N ratio correlated ($r = 0.81$, $P < 0.01$) with the log of total plate counts in acceptable samples of white and brown shrimp taken directly from fishing boats (Fig. 2). When spoilage odors became evident, after the samples had been stored on sterile ice, the TVN/AA-N ratio exceeded 1.5 mg N/mM. TVN/AA-N ratios of most spoiled samples appeared to form two distinct linear associations with bacterial counts. These values were connected with broken lines in order to indicate their linear relationship. The TVN/AA-N ratio for the freshly harvested shrimp did not correlate as well (evidence by the scatter) with total plate counts as did the ratio for shrimp which had been on ice for several days. As will be shown, the correlation between the bacterial count and the TVN/AA-N ratio may be indirect. (For future reference the TVN/AA-N ratio-bacterial regression line will be referred to as the enzymic line.)

In Figure 3 TVN/AA-N ratios of a number of commercial samples taken at the processing plant level are plotted against bacterial counts. Included are analyses of unfrozen brown shrimp and frozen shrimp (including peeled-deveined shrimp) of various species from different areas of the world. The quality of most of the samples used to obtain this data was very poor or unacceptable. From the odors evident, some of the samples had been treated with sodium bisulfite, which masks spoilage. Others probably had been treated, but treatment could not be verified. A number of samples with "acceptable" odors had bacterial counts $> 10^7$, TVN/AA-N ratios > 1.5 mg N/mM, volatile nitrogen levels > 30 mg N/100g (generally considered indicative of spoilage), a bleached appearance and sulfide odors in the trichloroacetic acid extracts suggesting spoilage and possible bisulfite treatment. Most TVN/AA-N ratios greater than those predicted by the enzymic line were grouped about two lines as in Figure 2. Three brown shrimp samples had high bacterial counts, but low TVN/AA-N ratios, suggesting recent massive bacterial contamination (the shrimp were known to be relatively fresh as boats had been at sea less than 5 days).

Some species of bacteria found on shrimp appear to produce volatile nitrogen more readily than others (Cobb and Vanderzant, 1971). This observation probably explains the grouping of TVN/AA-N ratios of spoiled samples into lines corresponding with certain bacterial levels. In samples where volatile nitrogen producing bacteria were not evident (evidenced by low TVN values), the TVN/AA-N ratios tended to follow closely the enzymic line.

Enzymic basis of test

Figure 4 illustrates experimental data (AA-N, TVN/AA-N ratios and bacterial counts) obtained from white shrimp during ice storage. TVN (not shown) and AA-N were variable while the TVN/AA-N ratio increased linearly until spoilage was evident (15 days). The sample taken at 15 days had a putrid odor with a soft mushy texture, suggesting proteolytic activity. Preliminary experiments indicate that freshly prepared TCA solution must be utilized for extraction. Otherwise, as illustrated in Figure 4, increasing AA-N

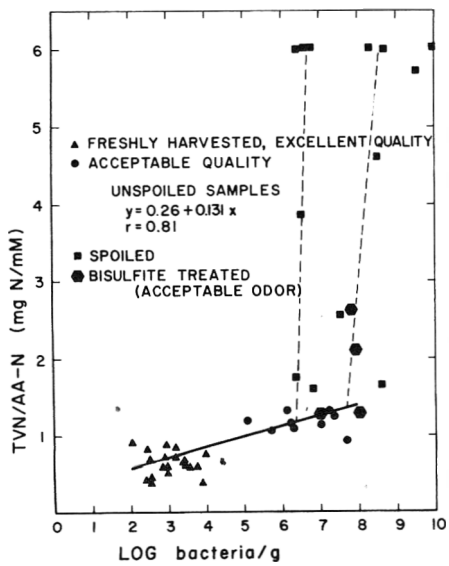

Fig. 2—Relationship between bacterial counts and TVN/AA-N ratio in white and brown shrimp taken directly from bay and Gulf waters and from fishing boats. Samples were kept on sterile ice and re-analyzed when putrid odors appeared. Only data from acceptable samples were used to calculate the regression line. Broken lines connect points which appear to have a definite relationship.

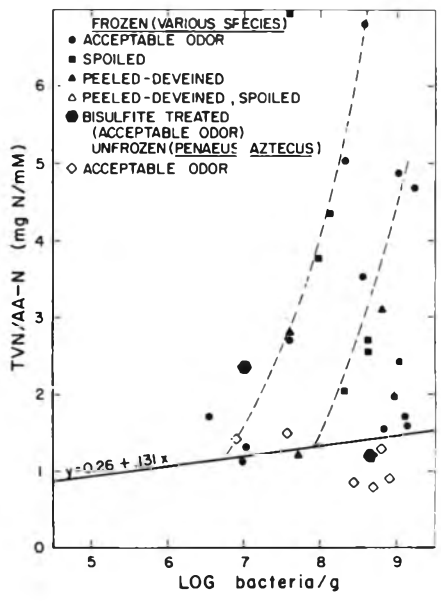

Fig. 3—Relationship between bacterial counts and TVN/AA-N ratios of multiple species of shrimp from many different areas of the world. Broken lines connect points which aopear to have a definite relationship.

levels (presumably due to unprecipitated peptides) tend to make the TVN/AA-N ratio unreliable as an index of quality when shrimp become soft and mushy after prolonged storage.

The use of the TVN/AA-N ratio to measure shrimp quality is based upon postmortem enzymic and bacterial volatile nitrogen production (Cobb and Vanderzant, 1971) and the corresponding loss of volatile nitrogen and amino acid nitrogen (Cobb et al., 1973b) as a result of drip and washing from melted ice. If the TVN/AA-N ratio in Figure 4 is plotted against logarithm of bacteria counts, the various points fall on or near the enzymic line illustrated in Figures 2 and 3. This suggests that the TVN/AA-N ratio and logarithm of bacterial count increase at approximately the same rate after the initial lag phase of bacterial growth.

Interference of glutamine and asparagine in TVN analysis

For a chemical test to be utilized by industry, it must be simple and reliable. A recent report (Cobb et al., 1973a) dealt with methods of TVN analyses which can be used to avoid production of extraneous ammonia during analysis. In Table 1 is presented the simulated microdiffusion analysis of a white shrimp extract. When the shrimp extract was treated for 4 hr with 7N NaOH, 0.98 mM/100g of ammonia was produced by hydrolysis. Approximately 70% of this came from the hydrolysis of asparagine and glutamine with the major portion being from glutamine. The source of the remaining ammonia has not been identified. Repeating this experiment with extracts from three other species of shrimp (*P. aztecus, P. vanami* and *P. occidentalis*) has given essentially the same results with the exception that 100% of the extraneous amonia came from hydrolysis of glutamine and asparagine.

Table 2—Production of ammonia from asparagine and glutamine during microdiffusion analysis with different releasing agents

Releasing agent	Asparagine[a] (%)	Glutamine (%)
7N NaOH	22[b,c]	25
Saturated Na_3PO_4	1	3
Na_3PO_4 · KOH	3	24

[a] Each solution contained 0.5 mg amide N.
[b] Average of six analyses
[c] 4-hr diffusion

In Table 2 is presented the effect during microdiffusion analysis of various releasing agents on the production of ammonia from asparagine and glutamine. Both asparagine and glutamine were hydrolyzed by 7N NaOH. Only glutamine was hydrolyzed to any extent by the Na_3PO_4 · KOH releasing agent, described by Cobb et al. (1973a). Saturated Na_3PO_4 did not hydrolyze asparagine and hydrolyzed less than 3% of the glutamine. Using saturated Na_3PO_4 as releasing agent, ammonia and trimethylamine distillation was not complete but a correction factor was determined by the use of ammonia standards.

A rapid method of TVN analysis by steam distillation has been developed (Cobb et al., 1973a). Asparagine and glutamine were extensively hydrolyzed (> 50%) during analysis by steam distillation when sufficient volume of distillate (approximately 10 ml) for accurate ammonia analysis was collected. The amount of hydrolysis was variable, depending upon the amount of distillate collected. However, as is evident in Table 3, steam distillation, using saturated Na_3PO_4 as releasing agent and properly controlled conditions, can give useful estimates of TVN contents.

Factors affecting use of the TVN/AA-N ratio to assess shrimp quality

Under certain conditions the individual components, TVN and AA-N, of the TVN/AA-N ratio may be more valuable in assessing shrimp quality than the ratio per se. When the washing action of ice is absent, AA-N levels in shrimp may increase more rapidly than TVN. In preliminary studies of packaged, refrigerated shrimp, increases in TVN contents > 30 mg N/100g and AA-N contents > 60 mM/100g have resulted in low TVN/AA-N ratios in spoiled shrimp. Where AA-N levels exceed 30 mM/100g, TVN levels > 30 mg N/100g indicate spoilage.

Pond (cultured) shrimp with high initial AA-N contents spoiled at 14–17 days, even though the ratios did not exceed 0.90 mg N/mM because of high AA-N contents (Vanderzant et al., 1973). In this case spoilage patterns differed greatly from those usually observed for wild shrimp. For such specialized cases,

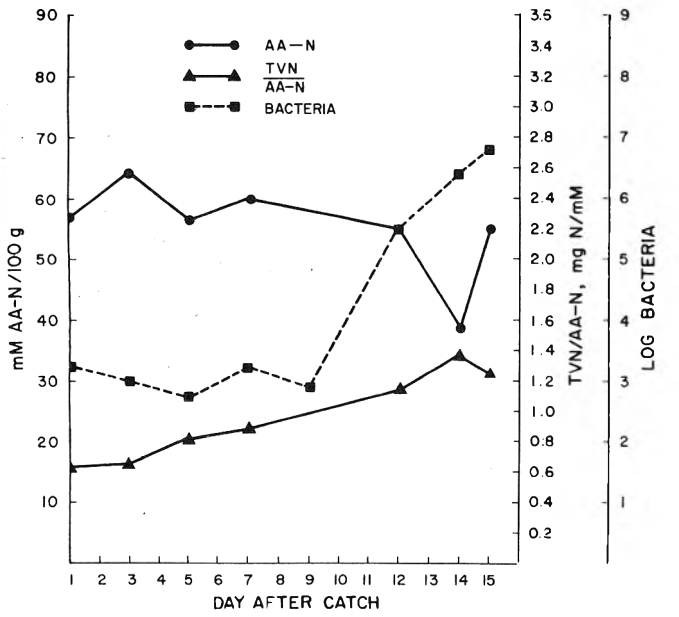

Fig. 4—TVN/AA-N ratios and bacterial counts of white shrimp stored in commercial ice.

Table 3—Comparison of different methods of TVN analyses on the TVN/AA-N ratio of different samples from a large lot of shrimp

Sample no.	TVN/AA-N (mg N/mM) Microdiffusion analysis 1.5 hr[a]	3.5 hr	Steam distillation	Log bacteria
40	1.43[b]	1.49	1.35	sp[c]
41	1.82	1.80	1.85	6.3
42	1.45	1.42	1.54	6.7
43	2.36	2.82	3.05	6.8
44q	1.81	2.01	2.28	sp
45	1.56	1.64	1.87	6.5
46	2.10	2.34	2.19	sp
47	3.51	3.65	4.05	6.5
48	1.40	1.36	1.33	6.7
mean	1.937	2.059	2.168	6.58[d]
SD	0.674	0.763	0.886	

[a] Diffusion time
[b] Average of three analyses
[c] Spreading colonies prevented count
[d] Geometric mean

spoilage values of the TVN/AA-N ratios may have to be adjusted.

The TVN/AA-N ratio may be a more accurate indicator of shrimp quality (organoleptic) than bacterial counts. In tests on frozen imported shrimp, some samples with TVN/AA-N ratios > 1.5 mg N/mM had bacterial counts ranging from 11,000–95,000 per g. These shrimp, which were from freezer boats, were rejected as spoiled by a trained taste panel.

Other factors which may affect the TVN/AA-N ratio, but not its usefulness as a quality indicator, are temperature and salinity of the water from which the shrimp were removed and bacterial contamination. Different water temperatures may cause differences in ammonia-producing enzymic levels and higher salinities may promote higher AA-N levels (Cobb et al., 1973b). Different species of bacteria, either natural flora or those induced by handling, grow at different rates and produce volatile nitrogenous compounds at different rates (Cobb and Vanderzant, 1971). This affects the usefulness of the test as a bacterial level indicator but not as a quality indicator.

From numerous tests and data presented in this and other communications the following guide lines for using the TVN/AA-N ratio are suggested:

(1)The most accurate analysis is TVN by the microdiffusion procedure with saturated Na_3PO_4 as releasing agent and AA-N by the copper procedure (Cobb et al., 1973a);

(2)For rapid TVN analysis either steam distillation with Na_3PO_4 as releasing agent or microdiffusion with Na_3PO_4 · KOH as releasing agent can be used (Cobb et al., 1973a);

(3)If the TVN content of the shrimp exceeds 30 mg N/100g (35 mg N if steam distillation is employed), the sample should be regarded as poor quality or spoiled regardless of the TVN/AA-N ratio;

(4)AA-N levels > 30 mM/100g, except for freshly caught shrimp, may indicate poor quality due to bacterial proteolytic activity;

(5)The TVN/AA-N ratio and the log of bacterial counts of "acceptable" quality shrimp have a high correlation; and

(6)Bacterial counts greatly exceeding those predicted by the TVN/AA-N ratio suggest recent, massive contamination.

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A TOTAL REDUCING SUBSTANCE TEST FOR ASCERTAINING OYSTER QUALITY

INTRODUCTION

THE DEVELOPMENT of rapid and accurate tests for the quality control of foods and food products is a very critical area in quality assurance research, especially when dealing with highly perishable foods which must be marketed soon after processing. These foods cannot be held for long periods of time awaiting final quality control results before distribution. Fresh oysters are such a food.

Many of the chemical quality tests which were developed for vertebrate fish have been suggested as chemical indicators for the quality of oysters and other shellfish, although their applicability depends to a large part on the particular variety of shellfish. Some of the tests which have been investigated include indole (Duggan and Strasburger, 1946), trimethylamine-nitrogen (Dyer, 1952), total volatile bases (Conway, 1957), ammonia nitrogen (Budd and Spencer, 1968), volatile reducing substances (Farber, 1952), and pH (Hunter and Linden, 1925; Pottinger, 1948; Abbey et al., 1957). Quite often these tests are accompanied by organoleptic and microbiological examination of the products.

The stress presently being placed upon the oyster industry by regulatory agencies (FDA and State Health Departments) to market an acceptable product necessitates the development of better analytical procedures than the slow and sometimes unreliable determinations now being used. This research was undertaken to develop a new rapid test for oyster quality which measures the group of compounds designated as total reducing substances (TRS). These TRS are derived as bacterial metabolic by-products during spoilage and are capable of reducing a solution of alkaline potassium permanganate.

EXPERIMENTAL

THE POTENTIAL usefulness of a total reducing substance (TRS) test for oyster quality was determined by comparison with previously available chemical indices, microbiological quality and organoleptic evaluations. Statistical analyses were used to show which tests correlated well with the TRS method.

Samples

Freshly shucked oysters used in these studies were obtained from a commercial packing plant in New Orleans, La. They were collected from beds near New Orleans and transported to New Orleans in a refrigerated truck at 4.4°C. They were shucked upon arrival by professional shuckers using facilities which conformed to FDA regulations (FDA Definitions and Standards for Shellfish, Title 21, Part 36, Section 36.10). After shucking, washing and draining, the oysters were packed in 1-gal cans, placed into ice chests, covered with ice and transported to L.S.U. Samples were procured in the Spring and Fall to account for seasonal variations.

The possibility existed that the chemical compounds which are used to measure oyster quality are affected by iced-storage. To ascertain if any changes did occur during a 15-day iced-storage period, duplicate samples were withdrawn from each of 2 gal of oysters for each season, at 0, 1, 3, 5, 7, 9, 11, 13 and 15 days. It was often thought that the results obtained with stored homogenates were a valid representation of what ensues in the whole oyster. However, preliminary investigations in this study and by Digirolamo et al. (1970) showed it inadvisable to generalize from homogenate studies to whole oysters. Therefore, testing was performed on whole oysters and, where applicable, the oyster liquor.

Total reducing substance determination

The use of TRS as a quality index for oysters was developed while experimenting with the volatile reducing substance (VRS) test of Farber (1952). The VRS test involves an aeration procedure in which air is circulated through a sample homogenate and then a standard alkaline potassium permanganate solution for a specific length of time, usually 45 min or 1 hr. The reduction of the permanganate by volatile compounds from the sample is revealed by a change from the purple potassium permanganate to the green potassium manganate. This change can then be measured either volumetrically or spectrophotometrically. Results are expressed as microequivalents of reduction per unit of sample. The results obtained with oysters using the VRS procedure were found to be extremely variable and the use of aeration equipment and the length of the aeration period prevented a large number of samples from being determined.

The measurement of TRS is essentially a modification of the VRS test in that it uses no aeration procedure. Either oyster meat homogenate or oyster liquor may be analyzed. Preparation of the homogenate consisted of blending 50g of drained oyster meat in a Waring Blendor for 3 min. 0.2 ml of homogenate was then pipetted into a 50-ml Erlenmeyer flask for testing. For testing the oyster liquor, 0.2 ml of liquor was pipetted directly from the container to the 50-ml Erlenmeyer flask. 10 ml of N/50 $KMnO_4$ in N NaOH was then pipetted into the reaction flask. From this point on, the procedure for both oyster meat and oyster liquor was the same except for the reaction time. Less reaction time was required for the oyster meat than for the oyster liquor. A time standardization procedure should be determined to ascertain this difference by plotting standard $Na_2S_2O_3$ needed for titration against reaction time (0–40 min).

After the reaction was essentially complete, 5 ml of 6/N H_2SO_4 was pipetted into the reaction flask. The contents were stirred and 3 ml of 20% KI was added. The liberated iodine was then titrated with N/40 $Na_2S_2O_3$, using starch solution as an indicator. The control for the unreacted permanganate was treated in the same manner as the sample.

The TRS concentration of the oyster meat or liquor was then calculated using the following formula:

$$\frac{\mu eq}{\text{per 0.2 ml of sample}} = \text{Titration for control} - \text{Titration for sample} \times 25$$

where: 25 = microequivalents (μeq) per ml of N/40 $Na_2S_2O_3$.

pH Determination

The pH of both the oyster meat and oyster liquor was determined by using a Corning pH meter model 10 with expanded scale. It was found that similar results could be obtained for oyster meat or liquor by using a 1:1 or a 1:10 dilution with deionized water. Due to the small amounts of oyster liquor available, the 1:10 dilution was used. Oyster meat homogenate was treated accordingly.

Trimethylamine-nitrogen determination

Trimethylamine-nitrogen (TMAN) was determined using the microisothermal distillation method of Beatty and Gibbons (1937) as modified by Dyer (1952) and Conway (1957).

Total volatile base determination

The procedure for determination of *total* volatile bases (TVB) was essentially the same as that for the determination of TMAN except that no formaldehyde was added to the isothermal distillation bottle.

Table 1—Total reducing substance results compared to chemical indices used for oyster quality evaluation[a]

Storage time (days)	Oyster meat					Oyster liquor			
	TRS[b]	pH[b]	TMAN[b]	TVB[b]	IN	TRS[b]	pH[b]	TMAN[b]	TVB[b]
0	135.5	6.69	4.16	19.7	6.32	108.3	6.97	4.72	15.2
1	136.2	6.57	9.73	22.6	5.73	117.5	6.74	12.60	30.2
3	136.3	6.49	12.96	28.3	5.42	127.4	6.60	12.34	28.6
5	138.7	6.32	14.95	32.1	5.70	130.7	6.49	14.43	35.2
7	142.1	6.24	11.26	27.2	5.25	134.6	6.40	11.81	33.1
9	141.4	6.21	13.46	27.8	5.07	136.5	6.28	15.22	38.8
11	143.4	6.11	15.00	28.1	6.05	137.7	6.22	16.27	39.9
13	144.0	6.05	13.45	28.8	5.60	137.9	6.13	13.38	40.9
15	144.6	5.96	13.02	30.1	6.62	138.2	6.02	14.43	42.0

[a] Codes for indices: TRS = total reducing substances (microequivalents of reduction per 0.2 ml of sample); TMAN = trimethylamine nitrogen (mg per 100g or ml of samples); TVB = total volatile base (mg per 100g or ml of sample); iN = indole (microg per 100g of oyster meat).
[b] Increases or decreases in indices values over the 15-day storage period were significant ($P < 0.01$).

Table 2—Bacterial counts on oyster meat and liquor during 15 days iced-storage[a]

Storage time (days)	Mesophilic[b]		Psychrophilic[b]	
	Oyster meat	Oyster liquor	Oyster meat	Oyster liquor
0	4.4	4.4	4.4	4.6
1	4.4	4.3	4.5	4.6
3	4.3	4.2	4.5	4.7
5	4.3	4.2	4.8	4.8
7	4.1	4.1	4.9	5.1
9	4.0	4.2	5.1	5.4
11	4.0	4.1	5.4	5.8
13	3.5	4.2	5.6	6.0
15	3.7	4.4	5.9	6.2

[a] Plate counts are expressed as the log of the number of organisms per 100g or ml of sample.
[b] Decreases in mesophilic plate count and increases in psychrophilic plate count were significant ($P < 0.01$) during the 15-day storage period.

Indole determination

Indole was determined by methods in the AOAC (1960) with two modifications: (1) a chloroform trap was used to collect the steam distillate, and (2) an additional 5 ml HCl wash (1:5) was used to clean the color reagent before reading in the spectrophotometer.

Microbiological plate counts

Microbiological plate counts were determined on both the oyster meat and oyster liquor using the following procedure: sample preparation for oyster meat consisted of homogenizing 50g of meat with 450 ml of sterile Butterfield's phosphate buffer (Butterfield, 1932) in a sterile Waring Blendor jar for 2 min; for oyster liquor, 1 ml of liquor was pipetted into a sterile 9 ml buffer blank; serial dilutions of the samples were then made and aliquots were plated in quadruplicate on Eugonagar (BBL); duplicate plates were incubated for 2 days at 32°C for mesophilic plate counts while duplicate plates for psychrophilic plate counts were incubated for 7 days at 5°C. Total colony counts were made with the aid of a Quebec Colony Counter.

Organoleptic evaluations

Organoleptic evaluations were conducted on raw, ice-stored oysters using both a hedonic scaling system (Peryam and Pilgrim, 1957) and a class designation system (Castell and Greenough, 1958). Panelists consisted of 12 laboratory personnel who had previous experience in organoleptic testing. On each test day, each panelist evaluated three oysters for odor, appearance, flavor and texture. Each of these characteristics was rated from 10 to 1 for the hedonic scaling with 10 representing the rating of excellent and 1 inedible. For the class designation each panelist rated the product as good quality (Class I), showing evidence of spoilage (Class II) or spoiled (Class III).

Statistical analysis and correlations

Data compiled from the chemical, microbiological and organoleptic tests were subjected to an analysis of variance and a determination of correlation coefficients. All analyses were conducted at the Computer Science Center, Louisiana State University, Baton Rouge.

RESULTS & DISCUSSION

THE AVERAGE VALUES for total reducing substances, pH, trimethylamine-nitrogen, total volatile basis, and indole are shown in Table 1.

The concentration of total reducing substances (TRS) increased uniformly in both oyster meat and liquor throughout the 15-day iced-storage period. These increases were highly significant ($P < 0.01$) at the 15th day. There would be less chance of a false positive due to experimental error or seasonal variation with analysis of the liquor since it exhibited a larger range of increase in TRS content than the meat. The range of values with the liquor is large enough so that a borderline or incipient spoilage stage would be sufficiently distinct from the fresh product to make its detection possible. Furthermore, the TRS concentration is more easily measured in the liquor than in the meat.

An analysis of variance showed highly significant differences ($P < 0.01$) between the TRS values for meat and for liquor. However, it was noted that there was no significant difference between oyster gallons on each test day or between replicates, showing that the TRS test gives results that are reproducible both within and between replicates.

The pH of both oyster meat and liquor decreased uniformly and significantly ($P < 0.01$) throughout the iced-storage period. The decrease is in close agreement with the observations of Piskur (1947), Pottinger (1951), Hoff et al. (1967) and Digirolamo et al. (1970). An analysis of variance showed that there was no significant difference between gallons within replicates. However, there was a significant difference ($P < 0.05$) between replicates which makes pH less reproducible than TRS.

No definite pattern could be established for trimethylamine-nitrogen (TMAN) content of either oyster meat or liquor during iced-storage. There was a general increase in TMAN content which was found to be highly significant ($P < 0.01$), but it was not consistent throughout storage. Similar changes in TMAN content of oysters have been reported by Lartigue et al. (1960). The ability of microorganisms to elaborate TMAN is decreased by iced-storage and by a lowering of the pH, thereby limiting the use of TMAN as an index of oyster quality (Castell and Snow, 1951). No significant differences were found between the TMAN content of oyster meat and liquor or between gallons within replicates. However, there was a highly significant difference ($P < 0.01$) between replicates which emphasizes another disadvantage in using TMAN as an index of oyster quality.

Table 3–Organoleptic evaluation of oysters during 15 days iced-storage[a]

Storage time (days)	Scores[b]				
	Odor	Appearance	Flavor	Texture	Average
0	9.3	9.4	8.8	9.2	9.2
1	8.9	9.1	8.2	8.2	8.6
3	8.1	8.1	7.6	7.6	7.8
5	7.5	7.8	6.7	7.1	7.3
7	7.1	7.5	6.5	6.7	6.9
9	6.2	6.4	5.8	6.3	6.1
11	5.9	5.9	4.8	5.6	5.6
13	5.4	5.1	3.6	4.4	4.6
15	5.4	3.9	2.5	3.1	3.5

[a] The values reported for organoleptic quality are expressed on a hedonic scale of 10 to 1 (10 = excellent, 6 = fair, 1 = inedible) and represent averages for 12 panelists in replicate determinations.
[b] The decreases in scores during the 15-day storage period were significant ($P < 0.01$).

Table 4–Correlation coefficients (r) of total reducing substances vs. chemical, microbiological and organoleptic data of ice-stored oysters

Total reducing substances (meat)	vs.	r
Total reducing substances (liquor)		+0.844**
pH (meat)		–0.942**
pH (liquor)		–0.937**
Trimethylamine-nitrogen (meat)		+0.414
Trimethylamine-nitrogen (liquor)		+0.419
Total volatile bases (meat)		+0.356
Total volatile bases (liquor)		+0.635**
Mesophilic plate counts (meat)		–0.545*
Mesophilic plate counts (liquor)		+0.056
Psychrophilic plate counts (meat)		+0.787**
Psychrophilic plate counts (liquor)		+0.751**
Cumulative organoleptic score		–0.846**
Total reducing substances (liquor)	**vs.**	**r**
pH (meat)		–0.876**
pH (liquor)		–0.927**
Trimethylamine-nitrogen (meat)		+0.308
Trimethylamine-nitrogen (liquor)		+0.287
Total volatile bases (meat)		+0.220
Total volatile bases (liquor)		+0.491
Mesophilic plate counts (meat)		–0.526*
Mesophilic plate counts (liquor)		–0.089
Psychrophilic plate counts (meat)		+0.545*
Psychrophilic plate counts (liquor)		+0.529*
Cumulative organoleptic score		–0.835**

* Significant at $P < 0.05$
** Significant at $P < 0.01$

A highly significant increase ($P < 0.01$) was found in the concentration of total volatile bases (TVB) in both oyster meat and liquor. There were fluctuations in the TVB content during the first week of iced-storage, but there was a uniform increase thereafter. There was a highly significant difference ($P < 0.01$) between TVB content of oyster meat and liquor. There were no significant differences between gallons within replicates, but the highly significant difference ($P < 0.01$) found between replicates is a distinct disadvantage in the use of TVB as an index of oyster quality.

Indole, determined only in the oyster meat, was detected in minute quantities throughout the storage period. There was a slight increase thereafter. This pattern did not contain any significant trends and is in agreement with the work of Duggan (1948) and Lartigue et al. (1960). The uselessness of indole as a quality index of oysters is further increased by the fact that significant differences ($P < 0.01$) occurred between gallons.

The results of the bacterial counts on the oyster meat and liquor are shown in Table 2. The decrease in the log of mesophilic plate counts of both oyster meat and liquor during the 15-day iced-storage period was highly significant ($P < 0.01$). These decreases closely parallel one another for the first 11 days of storage. After the 11th day, the mesophilic counts of oyster liquor increased slightly, while those for the oyster meat decreased more rapidly. The general decrease in the counts is in direct opposition to results reported by Rosen (1966) but is in apparent agreement with the work of Colwell and Liston (1960). There was a significant difference ($P < 0.05$) in counts between the meat and liquor, and highly significant differences ($P < 0.01$) between gallons within each replicate. There was no significant differences between replicates.

In contrast to the decreasing trend found with mesophilic counts, it was of interest to note that the psychrophilic plate counts of both oyster meat and liquor increased steadily during the storage period. These increases were highly significant ($P < 0.01$), as were the psychrophilic counts between meat and liquor and between gallons within each replicate. As with the mesophilic counts, there were no significant differences between replicates.

The difficulty in obtaining reproducible counts for either mesophiles or psychrophiles poses a definite disadvantage for their use as an index of quality. In addition, the times required for incubation seriously limit the use of microbiological counts as indices of oyster quality.

The decrease in organoleptic ratings for odor, appearance, flavor and texture during the storage period (Table 3) was highly significant ($P < 0.01$). The average scores obtained by grouping all of these characteristics was also found to be highly significant. The same trend was evident for all of the attributes, with flavor grading lower than any other throughout the storage period. There were highly significant differences ($P < 0.01$) for appearance and texture, and significant differences ($P < 0.05$) for cumulative quality between the two replicates. However, no significant differences were observed for odor or flavor between the replicates. This is important because odor and flavor are the most valuable organoleptic characteristics for evaluating results of various chemical tests. The strong possibility exists that some of the compounds measured could be contributory to odor or flavor.

The organoleptic panel graded the oysters as "good" from 0 through day 3 (Class I). From day 5 through 9, the oysters showed signs of spoilage, but were still organoleptically acceptable (Class II).

All panelists agreed that the oysters were "spoiled" (Class III) after the 11th day of iced-storage.

The correlation of TRS with other chemical and microbiological tests were of primary importance in this research in order to make an evaluation of TRS as an index of oyster quality. These correlation values which are found in Table 4 show that: (1) TRS (meat) correlates well with TRS (liquor), pH (meat and liquor), total volatile bases (liquor), mesophilic plate counts (meat), psychrophilic plate counts (meat and liquor) and cumulative organoleptic score.

The results of this investigation suggest that TRS content of either oyster meat or liquor could be used as an index of oyster quality. The pH of oyster meat and liquor also has merit as an index of oyster quality. Both the TRS test and pH compare favorably in several aspects: (1) they are both relatively rapid and simple to determine; (2) they are both reproducible, with TRS being more so than pH; and (3) no false positives were obtained with either test.

Trimethylamine-nitrogen and total volatile bases showed little merit as indices of oyster quality. Undoubtedly the organisms present in the product were not able to elaborate these compounds in large enough quantities under the environmental conditions. Although correlations were not made for indole, the lack of any definite trend in the indole content of oysters severely limits its use as an indicator of quality.

Organoleptic evaluations have several disadvantages, not the least of which is personal preferential judgements. However, the purpose of these evaluations was to gather information which could be used in determining correlations so that each objective test could be compared to subjective evaluations of oyster quality.

By virtue of the results of statistical analyses and correlations between the various chemical, microbiological, and organoleptic tests, it would appear that the total reducing substance test offers a rapid and reliable means of ascertaining the quality of ice-stored oysters. Of all the methods tested as indices of oyster quality, the TRS method most nearly approaches the criteria which a chemical indicator for food quality should possess.

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EFFECTS OF TEMPERATURE-TIME COMBINATIONS ON DONENESS AND YIELDS OF WATER-COOKED BROILER THIGHS

INTRODUCTION

LARGE QUANTITIES of further processed broiler parts are cooked and cooled in water. Commercial water cooking conditions of temperature and time vary according to type of cookers and coolers used. The majority of further processors use water temperatures in the range of 80–90.5°C with the exposure time set according to the temperature. This method contrasts sharply to most experimental procedures in which parts often are cooked by pressurized steam (Winter and Clements, 1957; Moran and Orr, 1969; and Moran et al., 1971) or in electric ovens set at various temperatures (Goodwin et al., 1962) with the parts wrapped in aluminum foil. From these procedures, variable results for yield and doneness have been reported. Because both doneness and final yield of the product are important, times and temperatures required to fully cook parts under commercial conditions should be investigated. Lyon et al. (1973), who cooked and cooled cut-up broiler parts in water, reported greater cooked yields than other investigators but measured doneness of the pieces only by end-point temperatures reached (internal piece temperature–85°C). They determined, however, that the optimum time and temperature required for cooked parts to reach a desired degree of doneness varied with the size and shape of the pieces.

Doneness has usually been evaluated by small untrained sensory panels or by observations of individual experimenters. As far as is known, criteria of doneness have not been established by correlating doneness scores of sensory panels with color meter readings.

There have been several reports in the literature recently concerning the color of cooked and uncooked poultry meat. Froning et al. (1968) used a Gardiner Model C-4 Color Difference Meter to examine the effects of age, sex and strain on turkey meat color. They reported that redness (high a_L values) of uncooked dark meat and of cooked dark meat from toms increased with age of birds.

Helmke and Froning, 1971, observed a decrease in Gardiner a_L values (redness) and an increase in L values (lightness) with advancing end-point temperatures in dark meat turkey rolls. In preliminary experiments with broiler parts cooked in water we found some degree of correlation between Hunter a_L values (redness) and various combinations of cooking temperature and time.

The purpose of this study was to examine the effects of combinations of cooking temperature and time on yield and doneness (color) of water-cooked broiler thighs.

EXPERIMENTAL

ICE-PACKED broiler carcasses (1021–1049g without giblets) were obtained from a local processing plant. Two thighs with back pieces were cut from each carcass with a circular saw. After any excess abdominal fat was removed, the pieces were weighed to the nearest gram.

Copper constantan thermocouples (30-gauge wire) were mounted on wooden cylindrical pointed probes (approximately 6.5 cm long). This allowed easy insertion and uniform placement of thermocouples. Probes were inserted in the pieces using the technique described by May et al. (1961). The probe was secured with a small wire around the distal end of the femur and the exposed portion of the probe. The individual pieces were then placed in plastic bags, identified by weight, and stored overnight at 4°C prior to cooking.

Pieces were cooked in 40-gal steam-jacketed kettles containing water agitated by a propeller set in the bottom of the kettles. The pieces were suspended in coarse mesh bags and placed in water at 90.5°C (kettle 1) until they reached a specified end-point temperature as determined by a 24-point recorder. They were then transferred to the second kettle (kettle 2) containing water at the specified end-point temperature and pieces were removed after the selected holding times. The pieces were then cooled in air-agitated, ice-slush until internal temperature reached 12.7°C. For zero holding time pieces were transferred directly from the first kettle (90.5°C) into the ice-slush.

After the thermocouple probe was removed, the piece was weighed, skin removed, pelvic girdle cut off and the distal end of the femur loosened using a knife to cut around the piece. An incision was made on the internal surface of the thigh adjacent to each side of the femur beginning at the proximal end of the femur and continuing to the distal end; the femur was then lifted and the meat underneath cut loose to free the bone and the narrow strip of meat resting on top of it. This left a skinless bone-out thigh piece with the ischiadic artery which paralleled the femur intact. The artery was exposed for examination by removing a series of muscle systems. The individual parts were then wrapped in aluminum foil, labeled and held at 4°C until they were ready for panel evaluation and Hunter Color Meter scoring later the same day.

Thigh pieces were visually evaluated for

Table 1—Statistical designs used to study end-point temperature-holding time combinations for doneness of water cooked broiler thighs

Experiment 1. Rotable surface response[a]		Experiment 2. Factorial design[b]	
End-point temp (°C)	Holding time at end-point temp (min)	End-point temp (°C)	Holding time at end-point temp (min)
73.8°	15	73.8°	0–30
76.1°	4.4 and 25.6	76.6°	0–30
80.5°	0, 15, and 30	79.4°	0–30
85.5°	4.4 and 25.6	82.2°	0–30
87.8°	15	85.0°	0–30
		87.8°	0–30

[a] Each temperature-time combination consisted of one thigh piece, except for 80.5°C–15 min which consisted of five pieces. The above combinations equaled one replication; and five replications were run.

[b] The end-point temperature holding times ranged from 0–30 min by 5 min increments. The above combinations with two thigh pieces per temperature-time combination equaled one replication; and two replications were run.

doneness characteristics by two highly trained panels, using color and doneness scales imposed on continua. Marks were then converted to numerical scores (0–60) by the use of a transparent overlay (Lyon et al., 1975).

After panel evaluations, the pieces were evaluated for color with a Hunter Color and Color Difference Meter (Model D-25-D) using the white plate (W783) as a standard. A 5-cm circular port was used and an optically inactive glass was inserted between the piece and the port to prevent "pillowing" of the sample into the port. The area surrounding the bone with the artery present was exposed in the port for color examination. Three color readings were taken per piece, L value (white to black), a_L value (red to green), and b_L value (yellow to blue).

A second-order rotatable response surface design (Cochran and Cox, 1957) was used in the first experiment. The end-point temperature holding time combinations are in Table 1. One thigh piece was cooked at each of the nine combinations, except at the center of the design, 80.5°C–15 min, where five pieces were prepared. This repetition provided an estimate of experimental error for testing the effects of end-point temperature and holding time. Each of the five replications consisted of 13 thighs. Responses from each thigh were Hunter L, a_L and b_L values, cooked yields and panel scores for color and doneness. Results from the 5 replicates were averaged and a second-degree model was fitted to the 13 points for each response.

To examine the end-point temperature-holding time region more thoroughly, a second experiment was run measuring Hunter a_L values and cooked yields only. A factorial design was used with 2 thigh pieces for each of the 42 possible combinations of six end-point temperatures and seven holding times (Table 1). Two replicates were run. Because of the manner in which the data were collected, the design of the experiment was that of a split-plot with end-point temperature as the main-plot and holding time at the end-point temperature as the sub-plot.

The term "holding time" is defined as the length of time that the thigh pieces were held in kettle 2 at one of the end-point temperatures, and should not be confused with total cooking time. The total cooking time was come-up time from placement of pieces in water at 90.5°C (kettle 1) until desired end-point temperature was reached plus holding time at that end-point temperature (kettle 2).

RESULTS & DISCUSSION

FIGURE 1 shows heating and cooling curves for Experiment 2. Initial temperatures ranged from 4–17°C and averaged 9°C. Differences were due to variation in the time at room temperature before cooking. This time usually ranged from 5–10 min depending on the time required to insert the thermocouple jacks into the recorder.

The heating portions of the curves (kettle 1–90.5°C) were very similar especially for the first 15 min when the temperature differential between the thigh pieces and the water was still large. The upper portion of the curves show the decreasing rate of temperature rise as differential between water and end-point temperatures narrowed.

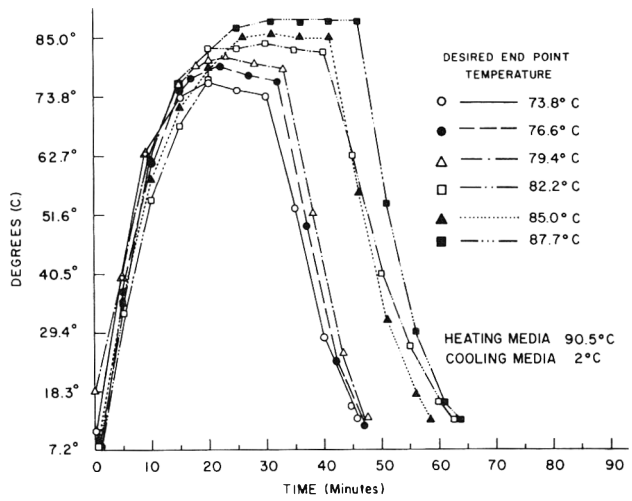

Fig. 1—Heating and cooling curves for the thigh pieces from the factorial design experiment with a 15 min holding time at the desired end-point temperature.

When the desired end-point internal temperature was reached, and the pieces were transferred to kettle 2, there was a slight increase in the internal temperature of the pieces due to equilibration of temperature between outer surface and internal positions. This phenomenon occurred for all temperature treatments, but the increase was directly related to the steepness of the heating curve at time of transfer.

Experiment 1

Table 2 shows the Hunter color values and the cooked yields from Experiment 1. Ranges of Hunter L and b_L values were narrow, and did not establish a definite pattern within the experimental temperature-time combinations. The Hunter a_L values ranged from 9.91 for 76.1°C, 4.4 min to 4.96 for 85.5°C, 25.6 min. As expected there was a general trend for the Hunter a_L values to decrease as both end-point temperatures and holding times were increased. The cooked yields in general showed expected decreases as the temperatures and times increased, although there was some variation within the same treatment. A part of the variation may have been due to the fact that weights were taken after the pieces were chilled to 12.7°C, and perhaps included a variable water uptake.

The replications at the mid-point temperature and time (80.5°C, 15 min) gave

Table 2—Hunter color values and cooked yields for the temperature-time combinations of the response surface design[a]

Temp (°C)–Time (min)[b]	Hunter L values	Hunter a_L values	Hunter b_L values	Cooked yields
73.8 – 15	51.45 ± 1.54	8.12 ± 0.98	14.32 ± 0.53	87.0 ± 2.13
76.1 – 4.4	48.97 ± 0.65	9.91 ± 0.63	14.18 ± 0.25	84.4 ± 1.02
76.1 – 25.6	50.40 ± 0.62	7.29 ± 0.29	14.10 ± 0.21	81.0 ± 0.91
80.5 – 0	50.66 ± 1.04	6.87 ± 0.60	14.36 ± 0.33	82.9 ± 2.14
80.5 – 15	49.65 ± 1.28	6.31 ± 0.28	13.96 ± 0.23	80.2 ± 1.55
80.5 – 15	49.11 ± 1.37	6.23 ± 0.24	13.92 ± 0.52	78.4 ± 3.71
80.5 – 15	49.18 ± 2.34	6.19 ± 0.41	14.40 ± 0.74	81.6 ± 1.14
80.5 – 15	49.73 ± 0.78	6.10 ± 0.14	14.55 ± 0.33	80.5 ± 2.66
80.5 – 15	48.81 ± 1.20	6.45 ± 0.29	14.00 ± 0.26	81.8 ± 2.43
80.5 – 30	47.90 ± 0.97	6.13 ± 0.36	13.64 ± 0.30	79.0 ± 3.76
85.5 – 4.4	50.39 ± 1.08	5.46 ± 0.23	14.15 ± 0.33	82.8 ± 2.78
85.5 – 25.6	52.20 ± 0.93	4.96 ± 0.35	14.78 ± 0.22	77.9 ± 2.73
87.8 – 15	49.42 ± 1.86	5.15 ± 0.19	14.20 ± 0.51	80.2 ± 1.46

[a] Each number in the table is a mean of five values with the standard error of the mean.
[b] Time represents minutes holding at designated temperature after reaching that temperature in a 90.5°C water bath.

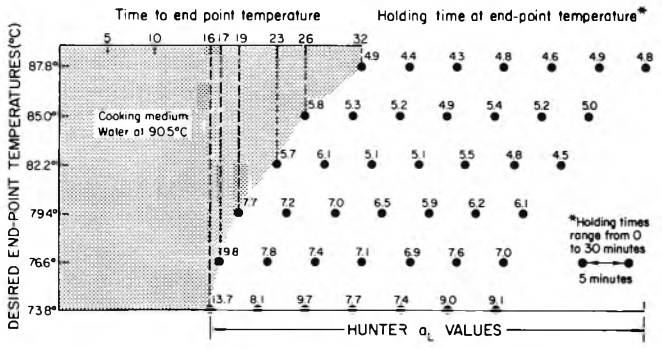

Fig. 2–Hunter a_L values for the temperature-time combinations of the factorial design.

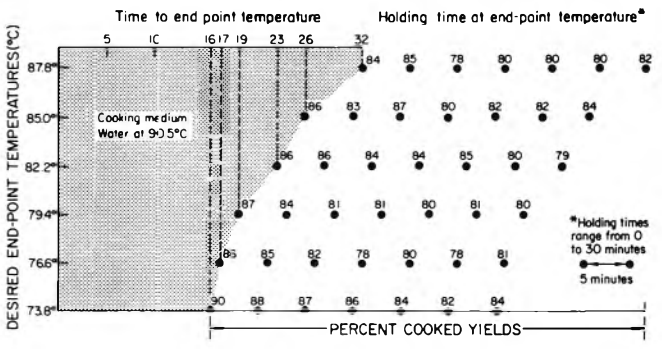

Fig. 3–Percent cooked yields for the temperature-time combinations of the factorial design.

an estimate of the experimental error for testing the effects of end-point temperature and holding time. Variation in Hunter a_L values for this temperature-time combination were small, ranging from 6.45–6.10. The cooked yields for 80.5°C, 15 min ranged from 81.8 to 78.4%.

The results of the regression analysis of data from Experiment 1 appear in Table 3. All responses, except Hunter L and b_L values, depended on holding time and end-point temperature of cook. The square of the multiple correlation coefficient R (between the response and the five independent variables in the model) gives the proportion of the variation among the 13 values of the dependent (or response) variable that is due to differences in end-point temperature and holding time. The high R^2 values for panel responses indicate that only a small proportion of the variation among panel responses was due to factors other than end-point temperature and holding time, such as individual panel variation or piece to piece variation.

Experiment 2

In Experiment 2, effects of holding time were compared more precisely than end-point temperature, and the analysis of variance has different error mean squares for these comparisons (Table 4). The partitioning of the sums of squares for end-point temperatures, holding times and their interactions was simplified by using orthogonal polynomials (Davies, 1956) made possible because of the equal spacings of the levels of temperature and time. This analysis includes the holding times as a factor, but not the "come-up" times to the desired end-point temperatures.

Temperatures and holding times significantly affected the Hunter a_L values of the cooked broiler thighs. The effects of these two factors were not independent as demonstrated by the significant temperature X time interaction (Table 4). Because differences between the two replicates were not significant and variation between duplicates within replicates was small, the four values from each temperature-time combination were averaged.

For cooked yield, only holding time had significant effects. There was an indication of a cubic end-point temperature trend, but this was not statistically significant in view of the large error mean square for testing temperature effects.

Figures 2 and 3 show the Hunter a_L values and the cooked yields at the temperature-time combinations of Experiment 2. The "come-up" times illustrate that as the temperature differential between the thigh pieces and the cooking media narrowed, a disproportionately greater time was required for the pieces to reach the desired end-point temperature.

Figure 2 shows a general decrease in Hunter a_L values for each temperature as end-point temperature holding time increased. The end-point temperature-holding time combination of 73.8°C–0 min produced the highest Hunter a_L values in the study. The other a_L values at 73.8°C exhibited an erratic pattern with increasing holding time. These thigh pieces had an internal color ranging from pink to bright red suggesting that color changes were beginning. The range of a_L values within the holding times for each end-point temperature narrowed rapidly as the end-point temperatures increased. The Hunter a_L values at 87.8°C reached a plateau between 4.9 and 4.3. This plateau effect indicated that the remaining amount of red color in the thigh pieces could not be removed by further water cooking in the experimental range of this study. The pooled standard error of the mean for a_L values in the second experiment was 0.52.

Table 3–Regression analysis of response surface design experiment

	Sum of squares			
Response	Regression (5 d.f.)	Error (7 d.f.)	Total (12 d.f.)	R^2 = Reg./Total[a]
L values	3.59	12.64	16.22	0.22
a_L values	18.92	1.84	20.76	0.91
b_L values	0.27	0.77	1.04	0.26
Overall color (Panel 1)	1151.97	53.92	1205.89	0.96
Doneness (Panel 1)	1185.71	47.53	1233.24	0.96
Artery color (Panel 2)	1959.85	92.88	2052.73	0.95
Meat color (Panel 2)	1361.17	107.26	1468.42	0.93
Doneness (Panel 2)	1830.58	79.95	1910.53	0.96
Cook yield	63.91	11.68	75.59	0.84

[a] R^2 = the proportion of the variation among the 13 values of the dependent variable that is due to the differences in temperature and time.

Table 4–Analyses of variance of Hunter a_L values and cooked yields

		Hunter a_L values			Cooked yields		
Sources of variation	d.f.	S.S.	M.S.	F.	S.S.	M.S.	F.
Replicates (R)	1	2.78	2.78	1.07 n.s.	118.52	118.52	1.36 n.s.
Temperature (T)	5	428.69	85.74	32.98 **	432.16	84.63	0.97 n.s.
Linear T_ℓ	1		395.66	152.18 **		92.90	1.06 n.s.
Quadratic Tq	1		27.05	10.40 *		23.42	0.27 n.s.
Cubic T_c	1		0.03	0.01 n.s.		295.28	3.38 n.s.
Remainder	2		2.98	1.15 n.s.		36.54	0.42 n.s.
Error 1 (R X T)	5	13.02	2.60		437.21	87.44	
Times (t)	6	68.11	11.35	10.32 **	694.54	115.76	11.30 **
Linear t_ℓ	1		35.22	32.02 **		536.71	52.41 **
Quadratic tq	1		22.96	20.87 **		127.52	12.45 **
Cubic t_c	1		5.44	4.95 *		6.33	0.62
Remainder	3		1.50	1.36 n.s.		7.99	
Temperature X Times	30	85.04	2.83	2.57 **	404.12	13.47	1.32
T_ℓ X t_ℓ	1		19.35	17.59 **		39.90	3.90
T_ℓ X tq	1		22.01	20.01 **		1.42	0.14
T_q X t_ℓ	1		0.00	0.00 n.s.		4.64	0.45
Remainder	27		1.62	1.47 n.s.		13.27	1.30
Error 2 (R x t & R x T x t)	36	39.49	1.10		368.67	10.24	
Duplicate Pieces	84	90.43	1.08		778.37	9.27	
Total	167	727.56			3224.59		

n.s. = Not significant
* Significant at the 5% level
** Significant at the 1% level

The cooked yields (Fig. 3) ranged from 90 to 78%, and in agreement with the first experiment, decreased in general as end-point temperature or holding time increased. Exceptions to these trends probably reflect the experiment variability noted in the first experiment. The pooled standard error of the mean for cooked yields in the second experiment was 1.6.

Longer exposure in kettle 1 (90.5°C) should have produced a lower cooked yield for the thighs at 0 holding time, but as Figure 3 illustrates this yield decrease was not proportionate to the increased time required for the pieces to reach the desired end-point temperatures. It was decided to do another experiment using the same end-point temperatures (Fig. 3) and 0 holding time with six thigh pieces at each end-point temperature. The cooked yields obtained were 91, 88, 89, 88, 85 and 84% for the end-point temperatures ranging from 73.8–87.8°C, respectively. These additional data reinforce the fact that longer cooking exposure did produce lower cooked yields but the decrease was not proportionate to cooking time as measured in this study.

The color data (Hunter a_L values) suggest internal end-point temperature ranging from 82.2–87.8°C with decreased holding time as the end-point temperature increases to water cook broiler thighs. thighs.

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SUBJECTIVE AND OBJECTIVE METHODS FOR ESTIMATING DONENESS IN WATER-COOKED BROILER THIGHS

INTRODUCTION

PRECOOKED and frozen convenience products account for a major portion of the poultry industry involved in further processing activities. Problems of adequate cooking and testing for doneness of broiler parts have become increasingly important in order to produce a product of acceptable quality while maintaining acceptable yield. At present, doneness of thigh pieces is used as an indicator of doneness of other broiler parts.

Criteria used to determine doneness of poultry are open to question. Published methods vary from rule of thumb procedures to recommendations of specific end-point temperatures. Most work to determine end-point cooking of poultry has been done with roasted turkey. Doneness of roasted turkey was determined by Alexander et al. (1951) by piercing the leg or breast muscle to determine tenderness and by manipulating leg and wing joints to detect softening of tendons. Cook et al. (1949) recommended a specific number of minutes per pound. Other workers have recommended specific end-point temperatures or a combination of time and internal temperature (Goertz et al., 1955). Lyon et al. (1973) observed that end-point temperature was an inadequate measure of doneness. Doneness appeared to vary with placement of the thermocouple, the size and shape of the piece and the method of cooking. Pieces considered to be undercooked usually exhibited varying degrees of pink or red color in areas around the bone.

Most work involving subjective determinations of doneness has been by individual observations of experimental workers. Goertz et al. (1960a, b) used small experienced but untrained panels to judge doneness of turkey before and after carving. Doneness was based on a scale of 7 (very overdone) to 1 (very underdone), the criteria for doneness being determined by the individual judge.

Some experimenters used color difference meters to measure color of cooked and uncooked poultry meat (Froning et al., 1968; Mugler et al., 1970; and Helmke and Froning, 1971). It therefore seemed feasible to attempt to establish objective doneness measurements by correlating sensory panel doneness scores with color values determined by instrumentation.

It is well recognized in the field of sensory evaluation that laboratory sensory testing is best performed by trained sensory panelists who function as a scientific instrument for correlating subjective responses with objective chemical or instrumental data. A relatively new sensory evaluation method, Quantitative Descriptive Analysis, has been introduced in sensory evaluation seminars held at the Stanford Research Institute (1972). The QDA approach uses profile methods (Amerine et al., 1965) for panel training and combines an elastic scoring system (Baten, 1946) with semi-structured scales (Raffensperger et al., 1956) for quantitating panel responses.

The purpose of this paper is to report the use of a subjective method (Quantitative Descriptive Analysis) and its relation to an objective color method used to determine criteria for the evaluation of doneness of water-cooked broiler thighs. An objective method supported by a subjective method for estimating doneness would be valuable in optimizing cooking conditions (time and temperature) for fully-cooked commercial poultry products.

EXPERIMENTAL

Cooking procedures

Thigh pieces used for panel training were cooked as described by Lyon et al. (1975), in water ranging from 71–93°C and for periods ranging from 15–90 min in order to provide a wide range of degrees of doneness.

The trained panels were evaluated by judgements on thighs cooked in 90.5°C water to internal temperatures ranging from 73.8–87.8°C, and then maintained at the end-point temperature for periods ranging from 0–30 min. A total of nine time-temperature combinations (Lyon et al., 1975) provided a range of doneness from extremely underdone to done.

Presentation of thigh pieces to panel

After cooking, thigh pieces were chilled in ice slush and deboned. The skinless bone-out thigh pieces, with the artery intact and the internal area around the femur exposed, were placed on individual coded white plates. Samples were presented randomly in specially designed compartmentalized observation booths painted a neutral gray and lighted by Chroma 55 fluorescent tubes providing 100 ft-c of light. Only one sample was placed in a compartment at a time to avoid obvious comparisons of the individual pieces. Each panel member judged a thigh piece, marked the score sheet, and went on to the next piece until all pieces in each session were evaluated. The same pieces in each session were evaluated by all members of both panels. Total evaluation time was less than 15 min and no changes were evident in appearance of the pieces as a result of time taken for evaluation.

Objective color measurements

A Hunter Color and Color Difference Meter (Model D-25-D) with a 5-cm circular port was used to evaluate the internal piece surface with the artery present. The white plate (W783) was used as a standard. Pillowing of the sample into the port was prevented by using an optically inactive glass plate between the sample and the port. L values (white to black), a_L values (red to green) and b_L values (yellow to blue) were taken per piece immediately after panel evaluation.

Panel training

Two panels, one of six and one of five members, were selected to participate in developing criteria for doneness evaluations through descriptive analysis training. We felt that comparison of the two panels undergoing similar training and evaluating the same thigh pieces would give an indication of the success and usefulness of subjective determinations of doneness.

Each panel trained separately for about 3 months, meeting 3 days a week for sessions of 30–90 min. It was stressed that training was necessary in order for panelists to function as a sensitive instrument and that personal preferences for the food product should be ignored.

Initially, panel sessions involved discussions of the problems evident in the varying appearances of thighs cooked by different time-temperature combinations. Several different criteria were suggested for doneness evaluations including color, moistness and fiber separation. Of these, color (primarily amount of pink or red) of the artery and of the meat in the area surrounding the bone was considered by consensus of the panelists to be the primary factor leading to initial rejection or acceptance of the piece as being done.

Rating scales of terms were developed to reflect gradations of color in relation to doneness. Panel 1 developed one rating scale for overall color of the meat and artery in the area around the bone. A doneness scale was then established by this panel based on these color evaluations

with primary emphasis on the color of the meat, and less emphasis on artery color.

Panel 2 developed two scales for color, one for the meat and one for the artery. This panel did not feel comfortable with the use of color terms and chose symbols to represent points on the scale indicating an arbitrary amount of red to brown (artery) and pink to tan (meat). The doneness scale developed was similar to that of Panel 1. However, doneness evaluations were based on artery color primarily and meat color secondarily.

To permit greater latitude than that provided by a set number of terms or symbols (rating scale points), continuum rating scales were constructed. Each continuum was represented by a horizontal 6-in. line with anchored points to represent rating scale terms or symbols (Fig. 1). Panelists then judged the thigh samples and recorded their evaluation by making a vertical line mark across the horizontal scale either on or between the anchored points that represented standards for which panelists were trained. The vertical mark was then converted to a score from 0 to 60, depending on its location along the 6-in. linear scale, by using a transparent overlay printed with a 6-in. line divided into 60 units.

Many of the training sessions involved evaluating thigh pieces under special lighting in observation booths designed for visual sensory evaluation work. Preliminary data for each training session were evaluated, and panel members were given feedback on their performance. When panelists seemed to differ considerably, round table discussions were held to determine solutions to the problems. Generally, these problems consisted of panel members forgetting their basic criterion for doneness (e.g., color instead of moistness, preference, etc.) and over-reacting to unusual pieces.

Statistical analysis

Correlation coefficients between response variables were calculated from data generated from a wide range of cooking temperature-time combinations. The correlation coefficients for each panel were also tested for homogeneity (Steel and Torrie, 1964). In addition, the relation between doneness scores and a_L values was tested by analyzing the degree to which the data could be fitted to a cubic curve, and regression equations were calculated for the central body of data.

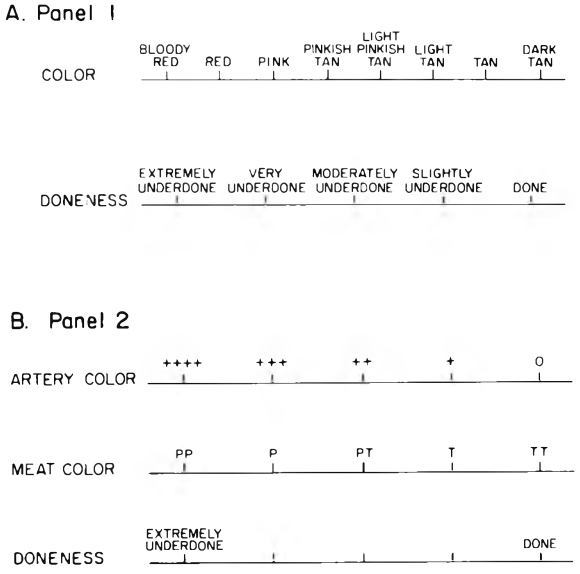

Fig. 1—Sample score sheets used by Panel 1 and Panel 2 for evaluating doneness of broiler thighs.

Table 1—Panel performance expressed as the correlation coefficient of individual doneness scores vs. observed Hunter a_L values

Panel 1 Panelist code	Correlation coefficient	Panel 2 Panelist code	Correlation coefficient
P_1	−0.836[a]	P_7	−0.810
P_2	−0.825	P_8	−0.769
P_3	−0.828	P_9	−0.776
P_4	−0.840	P_{10}	−0.684
P_5	−0.779	P_{11}	−0.805
P_6	−0.836		
X^2 =	1.407 NS[b]	X^2 =	3.261 NS
Pooled r =	−0.824	Pooled r =	−0.774

[a] Correlation coefficients must exceed 0.325 to be significant at the 1% level.

[b] An X^2 value must exceed 9.49 to be significant at the 5% level. A nonsignificant X^2 value indicates homogeneity of correlation coefficients.

RESULTS & DISCUSSION

Panel data, Hunter color data and their relationships

Panel performance was analyzed first by finding the correlation coefficient between individual doneness scores and observed Hunter a_L values (Table 1). All correlation coefficients were significant indicating a linear relationship between panel doneness scores and Hunter a_L values. Panelists displayed the ability to make discriminating judgements among thigh pieces with varying degrees of red color as measured by the Hunter Color Meter. Correlation coefficients for each panel were tested for homogeneity (Steel and Torrie, 1964). X^2 values of 1.407 and 3.261 for Panels 1 and 2, respectively, were not significant. Therefore, correlation coefficients for each panel group were considered to be homogenous, and the pooled r was −0.824 for Panel 1 and −0.774 for Panel 2, both highly significant.

Correlation coefficients for the response variables are shown in Table 2. The color evaluations determined by each panel were highly correlated with their respective doneness scores [Panel 1 = +0.956 and Panel 2 = +0.994 (artery); +0.944 (meat)]. This indicated that the respective panels did not need to use both color and doneness scales. Color evaluations per se could be eliminated and only doneness evaluations be used provided that doneness evaluations were based on the color of the cooked thigh piece rather than on other factors such as moistness, fiber separation or personal preference. Use of the color continua as part of the evaluation procedure, however, is recommended since they helped the panelists focus on the one basic criterion on which doneness evaluations are based, i.e., color.

The correlation coefficient for the doneness scores of both panels was +0.875 (Table 2). Despite this highly significant correlation, the doneness scores for the two panels were not in numerical agreement. This difference between panels in color requirement for doneness may be attributed to personality characteristics and individual preferences of

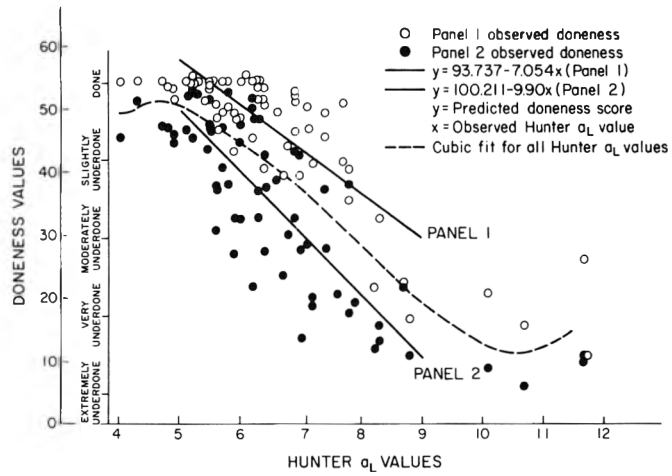

Fig. 2–Observed doneness related to Hunter a_L values and regressed doness for a_L values ranging from 5 to 9.

panel members. Hence concepts of doneness in relation to color were not exactly the same for each panel group even though the same training techniques were used.

In order to investigate further the relation between doneness scores and a_L values, the degree to which the data could be fitted to a cubic curve was tested by computer. This cubic relation (Fig. 2) reinforces the observation that both panel groups were not discriminating between samples of different a_L value in the ranges of extreme a_L values. Hunter a_L values greater than 9 represented "extremely underdone" pieces and a_L values less than 5 (Panel 2) and 6 (Panel 1) were "done." Therefore, values <5 and >9 were eliminated from analysis and a linear regression fitted to the data between these points. Doneness scores and a_L values from each panel showed a significant linear relationship. The confidence interval for individual panel scores of Panel 2 was 18.5–54.5 at the midpoint. The confidence interval at the midpoint for mean of the panel scores was considerably smaller, 35.6–37.6. Doneness estimates from Hunter a_L values in the range 5–9 would not be highly discriminatory when based on individual panel scores. However, a significant difference of 0.6 in Hunter a_L value could be detected if consideration was based on means of panel scores in Figure 2.

The slopes of the regression lines for the panels were significantly different indicating that the two panels differed in their response to the same change in a_L value. The steeper slope for Panel 2 shows this panel assigned greater changes in doneness than Panel 1 for the same change in a_L value. The vertical displacement between the two regression lines also points out lack of agreement between the two panels on their assignment of degrees of doneness to the same samples.

Doneness scores from Panels 1 and 2 had correlation coefficients of –0.361 and –0.810 with Hunter a_L values, respectively. Scores of 43 and 55 on the doneness scale corresponded to "slightly underdone" and "done." Hunter a_L values of 7.1 and 5.5 corresponded to "slightly underdone" and 5.2 and 4.0 to "done" scores for Panels 1 and 2, respectively. Therefore, the panels failed to establish an absolute level of doneness in any sample. This is understandable, because level of doneness is a matter of definition which involves an element of preference, and was not established by the small panels even though they were highly trained. The correlations and regression equations established in the study for two trained laboratory panels could be extended to consumer panels, replicated in different geographic areas and for different ethnic groups.

These results re-emphasize the need for an objective method to measure the complex factor of doneness. Based on the data from this study, measurement of Hunter a_L values provides an objective method to monitor standards of doneness in pre-cooked poultry products. These data also suggest that further work to establish an absolute level of doneness might be confined to Hunter a_L values ranging from 4.0–7.1 since this range of Hunter a_L values corresponded to panel doneness scores of 55 (done – Panel 1) and 43 (slightly underdone – Panel 2), respectively.

The descriptive analysis approach proved to be a beneficial method of panel training for such a complex sensory factor as doneness. By involving panel members in the decision-making, a more complete understanding of the sensory evaluation process was recognized and high levels of interest and cooperation among panelists were aroused. Use of the continuum rating scales imposed on 6-in. lines allowed panelists the freedom to judge adequately samples that did not correspond exactly to the standards with which they were trained.

Based on these findings, the subjective and objective methods for estimating

Table 2–Correlation coefficients between response variables[a]

	Hunter a_L	Hunter b_L	Overall Color Panel 1	Doneness Panel 1	Artery Color Panel 2	Meat Color Panel 2	Doneness Panel 2
Hunter L	–0.326	0.698	–0.163	0.008	–0.046	–0.213	–0.079
Hunter a_L		–0.138	–0.780	–0.861	–0.820	–0.742	–0.810
Hunter b_L			–0.106	–0.025	–0.093	–0.138	–0.091
Overall Color – Panel 1				0.956	0.846	0.950	0.881
Doneness – Panel 1					0.856	0.891	0.875
Artery Color – Panel 2						0.911	0.994
Meat Color – Panel 2							0.944

[a] Hunter values are individual values for each piece in each replicate. Sensory values are mean panel scores for each piece in each replicate. For significance at the 5% level a coefficient must exceed 0.25.

doneness in cooked poultry will be extended to cover other cooking methods. Future work will attempt to establish a definitive level of doneness from sensory and objective data. This will involve extensive consumer testing which will also focus on sensory factors other than color, such as moistness, texture, taste, etc.

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INHIBITION OF Aspergillus niger IN AN INTERMEDIATE MOISTURE FOOD SYSTEM

INTRODUCTION

THE EFFECT of water activity (a_w) on the growth of microorganisms has been reviewed by Scott (1957) and Troller (1973). It is known that as the water activity (a_w) decreases, the growth of microbes is slowed or prevented. Recently, intermediate moisture foods (IMF) having a moisture content of 15–40% H_2O and an a_w of 0.65–0.85 have been examined in terms of microbial stability (Hollis et al., 1969; Labuza et al., 1972). Microbial spoilage in foods of this type is due primarily to mold or yeast growth since most bacteria do not grow at the lower a_w.

Pet foods are examples of commercial IMF meat products. During the processing of these semi-moist foods, which includes extrusion cooking, the incident yeast and most vegetative bacteria are killed, but the mold spores survive. Since these foods have a relatively high pH (pH 5–6) and are stored at room temperature, the mold spores are a potential spoilage problem. Thus, mycostatic agents are incorporated into the food product to prevent growth and thus extend the shelf life of the product.

The growth of mold (as well as other microbes) is dependent on a_w, temperature, atmosphere and substrate, i.e., pH, nutrients and inhibitors. At a given a_w, the growth response will change if any of the above factors are suboptimal (Christian, 1963). The effectiveness of a mycostatic agent should be greater in the IMF a_w range than in high moisture foods. The mycostatic system used in most IM meats is a combination of 2.0% propylene glycol and 0.3% potassium sorbate (w/w) as described by Kaplow (1970). The purpose of this storage study was to test the variety of common food additives for their ability to inhibit the growth of *A. niger* on an intermediate moisture food.

MATERIALS & METHODS

Food systems

The intermediate moisture food used was an adaptation of Pemmican, an old Indian trail and winter storage food made of buffalo meat and berries. Chicken was chosen as the base for the IMF used in this study. The composition of the basic system is shown in Table 1.

The a_w of 0.85 allowed the growth of the mold but inhibited the growth of the few natural bacterial contaminants in the raw materials. No yeast contaminants were found. A cold-mixing procedure, as shown in Figure 1, was used to prepare the Hennican (the name given to the chicken based IMF). The unsalted, hulled peanuts (Skippy Co., Minneapolis, MN) and the chicken (Aslesen's Banquet Table, canned deluxe boned chicken meat, #3022) which was freeze dried were finely ground separately in 1/2-pt glass blender jars in an osterizer blender. The nonfat dry milk and all other dry ingredients were mixed separately. The raisins (dried seedless) were ground in a Hobart food chopper and then blanched in a microwave oven for 1.5 min to destroy the enzymes responsible for enzymatic browning. The dry components, ground raisins, peanut butter (creamy style), honey and sterile distilled water were kneaded together in a 500 ml Brabender Farinograph bowl at fast speed for 5 min to achieve a workable paste. This mixture was divided into two equal parts and citric acid, 2% (w/w), was mixed into one part for 3 min in the Farinograph bowl. The food additives were added via the appropriate carriers to the systems with and without citric acid, and a spore suspension of *A. niger* was added to all systems. 3 min of mixing was found adequate for each addition. All systems were then shaped into rectangles weighing approximately 2.5g each by rolling out the paste and cutting the pieces to 2 cm × 1.5 cm × 0.5 cm. Two samples were placed into sterile plastic petri dishes (60 × 15 mm) and then all samples were stored in desiccators without vacuum at 23°C over a saturated solution of $LiSO_4$ (a_w = 0.85 at 22°C). No weight change occurred during storage showing equilibrium. The cover was removed periodically so that oxygen was not limiting.

Additives

The additives tested fall into four main categories: acid-type, parabens, an antibiotic, and polyhydric alcohols. Of the acid-type microbial inhibitors, potassium sorbate is commonly used in cheeses, breads and intermediate moisture foods to prevent mold and yeast growth. Calcium propionate is used in cheeses and yeast-leavened breads to inhibit molds and bacteria. (It does not interfere with the fermentation activity of yeast.) Benzoic acid inhibits molds and yeast and is used in foods of low pH where bacteria normally do not grow.

The acid-type inhibitors must be in the undissociated form to be inhibitory to microbes (Sauer, 1972). Chichester and Tanner (1968) suggest that the reason these preservatives are effective only in the undissociated form is because in that form it is highly lipid soluble and accumulates in the lipid structures of cells. There it somehow inhibits normal cell metabolism. However, the mode of action of these preservatives is still not known.

The parabens are widely used in foods and cosmetics as antimycotic agents. Propyl paraben is more effective than the methyl ester but is less soluble in H_2O. To achieve the best effect the esters are often used in combination (Chichester and Tanner, 1968).

Of the antibiotics, pimaricin (natamycin) is an effective inhibitor of mold and yeast and is used in very low concentration as a dip solution for cheeses and sausages in some European countries (Clark et al., 1964). On agar, pimaricin inhibits *A. niger* at 5 ppm (Klis et al., 1959).

1,3-butanediol (Celanese Chem. Co.), a polyhydric alcohol, is used in foods as a flavor carrier. Mannitol, sorbitol and glycerol are used in foods as humectants, plasticizers or sweeteners. The additives were mixed into the food via a water or acetone carrier, depending on the solubility of the additive. When water was used, water had been omitted from the formulation of that particular system to prevent an increase in a_w. When acetone was used the food system was kneaded until the solvent odor disappeared (an additional 5 min). The mold spores were then added.

The mold used in this study was chosen since it is a frequent contaminant of commercial intermediate moisture foods. *A. niger* (Plant Pathology, University of Minnesota) was streaked onto a sterile cotton-plugged 150 ml prescription bottle slant containing TSYA (Trypticase soy agar and 0.5% yeast extract, BBL brand). The culture was grown at 23°C for 5 days. The mold spores were washed from the slant with 30 ml of sterile phosphate buffer (0.125%) and the turbidity of the suspension was measured to estimate the mold spore concentration. The spore suspension was subsequently diluted and 1 ml was inoculated into the mixing bowl to give an initial mold spore count of 1×10^4 CFU (colony forming units)/g food. The amount of water used as a vehicle for

Table 1—Composition of IM food: Hennican[a]

Component	Amount (wt basis)
Raisins	30%
H_2O	23
Peanuts	15
Chicken (freeze-dried)	15
Non-fat dry milk	11
Peanut butter	4
Honey	2

[a] Moisture content = 41g H_2O/100g solids; a_w = 0.85

the mold spores was omitted from the formulation of the systems to maintain the desired a_w of 0.85.

After 3 min mixing in the Brabender bowl, samples of the systems were plated to determine the initial viable mold count. 5g of the food was blended with 45 ml of sterile deionized water for 1 min and TSY agar plates were used in duplicate at 23°C for 3 days.

The pH of the systems was determined by two methods. A direct reading was taken by pressing a nonaqueous Beckman electrode (#39142) into the squares of food. The gran plot method of Labuza (1974a, b) was also used. To 3.0g of food either 1, 2 or 3 ml of distilled deionized water was added and stirred in to make a slurry. The pH was read after 5 min equilibration. The pH was plotted against the grams of H_2O added on gran plot paper (100% volume-corrected, Orion cat. no. 900093). The value at zero addition is the pH. This method is useful for IMF systems and was found more reliable than the method recommended in the AOAC book of standard methods (AOAC, 1970). The two methods used in this study were found to give the same pH value within ± 0.05 pH units which is the probable variation in composition.

The water activity (a_w) was measured by a manometer technique (Labuza, 1974a, b). The technique has an accuracy of ± 0.005 at an a_w of 0.85. Storage of the samples over the saturated salt solution made certain that this a_w was constant throughout storage.

The moisture content of representative duplicate samples of the systems with and without citric acid was determined by the vacuum oven method at 29 in. Hg and 60°C for 24 hr.

RESULTS & DISCUSSION

THE PARAMETERS and results of this study are shown in Table 2. The criterion for no inhibition was when mold became visible. This could indicate a consumer acceptance criterion. As should be expected, all the acid-type inhibitors were completely effective at pH 4.2 showing no growth for over 9 months in this intermediate moisture food. With a pH in the normal range for meat products, 0.3% K-sorbate is an effective mold inhibitor without the added effect of propylene glycol. If the food were higher in pH, more K-sorbate than the FDA allowance would be necessary. A similar trend is found for the propionate. Benzoic acid is not effective in the amount allowed by FDA restriction (0.1%) at the higher pH.

The parabens inhibited the mold at all levels tested. As seen, a lower concentration than found for the acid-type inhibitors is effective. The antibiotic, pimaricin, is effective at 0.002% (or 20 ppm) at both pH 5.7 and pH 4.2. Klis et al. (1959) found inhibition at 5 ppm in agar at pH 5.6. However, they only incubated for 2 wk. It is possible growth might have occurred after that time. From a shelf-life testing standpoint, a longer time should be used. This study found 10 ppm to be ineffective. Most likely the antibiotic was not distributed as well in the heterogeneous food of this study.

The polyhydric alcohols, including propylene glycol, were inhibitory at lower concentrations compared to the amount allowed for various uses by the FDA. The minimum inhibitory concentration determined for 1,3-butanediol

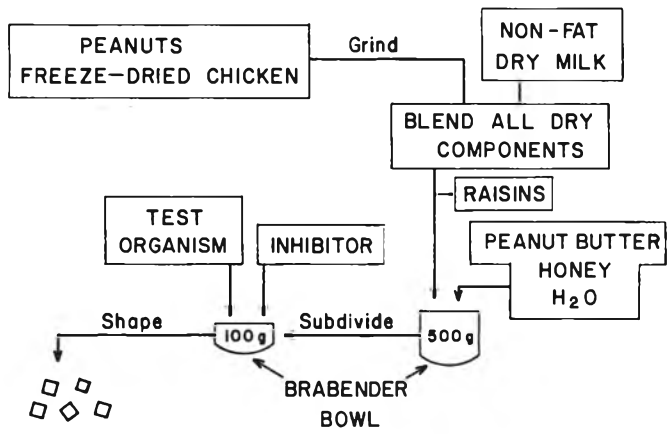

Fig. 1–Cold-mixing procedure used to prepare Hennican, the chicken-based IMF used.

Table 2–Microbial inhibitors in Hennican, a_w 0.85

		Time for 1st appearance of A. niger[a] (wk)	
Inhibitor	**%w/w**	**pH 5.7**	**pH 4.2**
Potassium sorbate	0.15	2	ng
	0.30	ng	ng
Calcium propionate	0.1	2	ng
	0.2	19	ng
	0.3	ng	ng
Benzoic acid	0.2	7	ng
	0.3	ng	ng
Methyl paraben	0.03	ng	ng
	0.05	ng	ng
	0.10	ng	ng
Propyl paraben	0.01	ng	ng
	0.03	ng	ng
	0.04	ng	ng
Parabens Me/Pro (2:1)	0.05	ng	ng
	0.10	ng	ng
Pimaricin	0.001	1	4.5
	0.002	ng	ng
	0.005	ng	ng
1,3 Butanediol	1.0	1	22
	2.0	ng	ng
	4.0	ng	ng
Propylene glycol	1.0	ng	ng
	2.0	ng	ng
	4.0	ng	ng
Mannitol	1.0	ng	ng
	2.0	ng	ng
Sorbitol	1.0	ng	ng
	2.0	ng	ng
Glycerol	1.0	ng	ng
	1.0	ng	ng
Control		1	4.5

[a] 9 months storage at 23°C; ng = no mold growth during the period of storage

(2.0%) is below the inhibitory concentration found by Frankenfeld et al. (1973) in studies of *A. niger* on food systems of higher a_w, and similar pH.

The interaction of a_w, solute used and pH in their effect on microorganisms has been reported by many workers (Troller, 1973). The solutes used to lower a_w are often polyols, such as glycerol, propylene glycol, 1,3-butanediol, and it is certain that their inhibitory effect is not entirely related to their water binding capacity; however, the reason for their toxicity is not known. Working with *Neurospora sp.*, Charlang and Horowitz (1971) found that glycerol was less inhibitory as compared to NaCl or sucrose at the same a_w. They suggest the difference is due to the solute's electrolytic properties. They found that at low a_w, a substance essential for spore germination was lost to the medium and when the substance was isolated and supplied to the spores, germination occurred. They suggest that the release of this substance is due to osmotic effects which are related to the permeability of the cell to a solute. Solutes such as glycerol, which easily entered the cell preventing osmotic imbalance, did not inhibit germination as much.

Webb (1960) suggested that death at lowered a_w was due to the dehydration of an essential macromolecule. He suggested that if the solute had a hydrogen bonding ability, it may bind on the macromolecule and prevent denaturation from loss of the hydration shell as a_w decreases. This could explain why glycerol was less toxic than NaCl in the Charlang and Horowitz (1971) study, however, it does not explain the toxicity in this study.

Horner and Anagnostopoulos (1973) studied the growth rate of several molds as a function of pH, a_w, temperature and the solute used to adjust a_w. They found glycerol to be more inhibitory to *A. niger* than sucrose at the same pH and a_w. On agar at a_w 0.86 and pH 3.7, growth of *A niger* was visible on media containing glycerol as the humectant after 5 days at 25°C. This is a very short induction time compared to the present study in which the control Hennican (no glycerol added) at a_w 0.85, pH 4.2 didn't show growth of the mold for 4.5 wk. Under the stress presented by this food system as compared to nutrient agar, the additional adverse effects of only 1% of glycerol was enough to completely inhibit the mold for over 9 months.

The mode of action of these inhibitors is not known, but they are effective inhibitors of the test organism in this study at suboptimal pH and a_w. This study will be extended to other molds commonly found as contaminants at low a_w and to the pathogenic bacteria *Staphylococcus aureus.*

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COMPOSITIONAL AND METABOLIC GROWTH EFFECTS IN THE BOVINE
Muscle, Subcutaneous and Serum Fat Classes

INTRODUCTION

RECENT consumer concern over the presence of fat in foods, especially in meat products, has precipitated a need for a better understanding of biological controls of the deposition and composition of animal fats.

It has long been recognized that the amount and composition of lipids is not uniform throughout the animal body. This variability is apparently the result of several determinants such as sex (Terrell et al., 1969; Gillis et al., 1973), breed (Cramer and Marchello, 1962, 1964; Gillis et al., 1973), season (Cramer and Marchello, 1964; Link et al., 1970b, c) and body location (Blumer et al., 1962; Terrell et al., 1967; O'Keefe et al., 1968; Moody and Cassens, 1968). Interrelated with these factors is the effect of growth. Investigations in this area (Callow, 1948; Andrews, 1958; Zinn, 1967) have demonstrated the existence of a definite order of deposition among the various body fat depots in response to growth and fattening (i.e., visceral fat is deposited first, marbling fat last). More detailed studies (Link et al., 1970a, b, c; Waldman et al., 1968) have revealed an alteration in the composition of these adipose depots with increasing age, such as an increase in total intramuscular neutral lipids, an overall increase in the unsaturation of subcutaneous lipids and several significant changes in the fatty acid profile of muscle lipids.

The majority of these studies, however, have not investigated changes in fat composition occurring during the very early stages of growth. If the composition of beef fat is to be controlled or modified to fit consumer demands it is important that these early changes be elucidated.

This investigation was conducted to determine when and to what degree the changes in muscle, subcutaneous and serum fat classes occurred during the growth of cattle from 28 days of age to slaughter.

EXPERIMENTAL

Test animals

The study involved 27 animals of Hereford (8 steers and 3 heifers), Angus (4 steers and 3 heifers) and Holstein (5 steers and 4 heifers) breeding. Calves were placed on the experiment at 28 days of age and were maintained on typical grower and finisher rations throughout the trial. They were individually fed and housed for the duration of the study and were slaughtered on a constant finish basis at an estimated choice grade. Slaughter age ranged from 420 to 602 days of age.

Sample collection

The biceps femoris muscle was chosen for biopsy and analysis because it is of sufficient size to withstand sampling, is easily defined in the live animal regardless of age, and it possesses a reasonable correlation with the muscle mass of the animal. The muscle biopsies were taken in an alternating fashion between the right and left sides at 28 days of age, 56 days of age and at 56-day intervals thereafter until the animals were slaughtered. Since anatomical location has been shown (Blumer et al., 1962; Terrell et al., 1967; O'Keefe et al., 1968; Moody and Cassens, 1968) to have a marked influence on the lipid content of a given muscle, the biopsies were performed at approximately the same location in the muscle between calves for each period.

After removal, muscle samples (7–10g) were minced, dried overnight in a vacuum oven and extracted on a Bailey-Walker apparatus. After extraction the solvent was evaporated under nitrogen and mild heat. The resulting lipid samples were stored under nitrogen (−30°C) until analyzed.

Subcutaneous fat samples were obtained from the tail-head region because it proved to be a reliable source of fat during the early development of the calf and consequently were continued throughout the study. Samples (5–8g) were treated in a fashion similar to the muscle.

Blood serum samples were collected every 28 days via jugular vein puncture. The serum lipids were extracted as described by Thrall (1969).

Lipid analysis

Classes of lipids were determined using thin-layer chromatography (TLC). The plates (20 cm × 20 cm, Brinkman Instr. Inc.) were developed using a double development system. During the initial step the solvents (40–60°C boiling range petroleum ether, diethyl ether and glacial acetic acid in a ratio of 85:15:1.5) were allowed to ascend half way up the plate. After adequate drying a second solvent system, consisting of below 40°C petroleum ether and diethyl ether (94:6), was permitted to ascend to about 1.5 cm from the top edge of the plate. All solvents were redistilled prior to use to insure purity. The plates were visualized by first dipping in a 10% (by weight) phosphomolybdic acid and distilled water solution. After drying they were charred in a 200°C oven for 4–6 min. Quantitative estimation of the lipid classes was performed densitometrically, using a Photovolt Densicord (model 52C), complete with strip chart recorder fitted with a disc integrator.

Data analyses were conducted using the

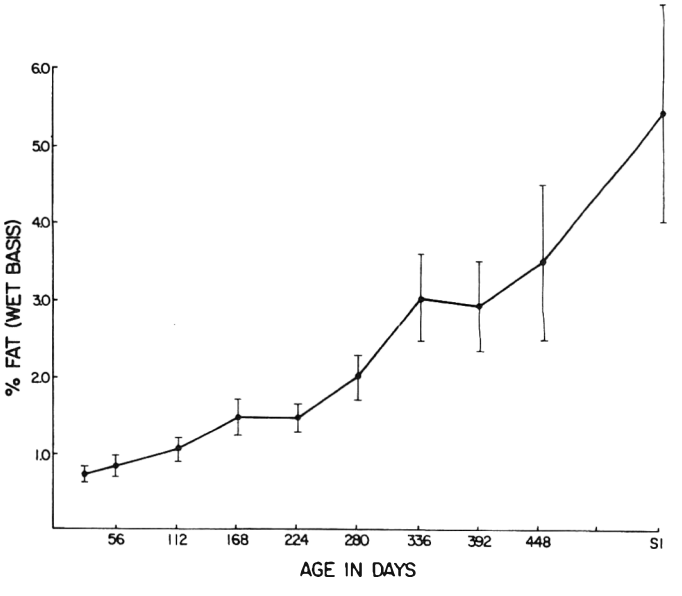

Fig. 1—Average intramuscular fat content of muscle biopsies (95% confidence intervals).

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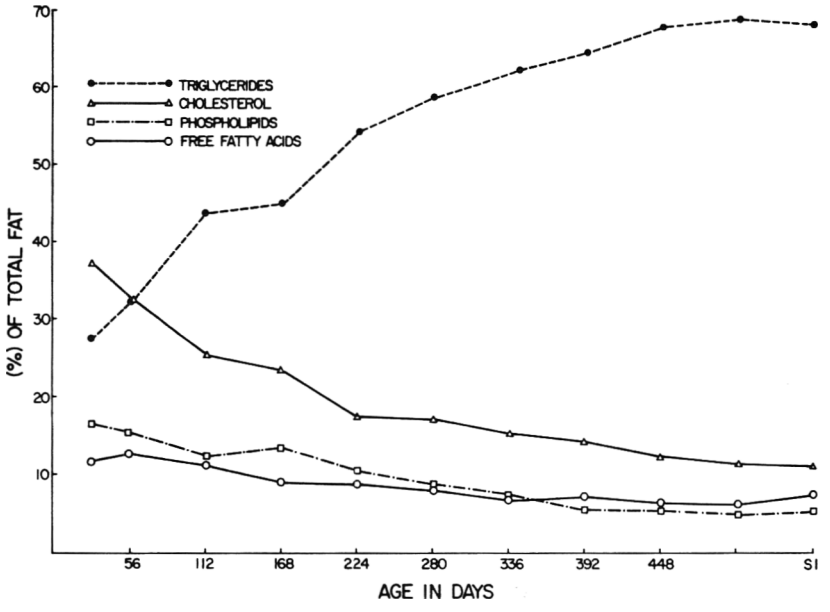

Fig. 2–Growth changes in primary intramuscular fat classes.

least squares procedure for analysis of data with unequal subclass numbers (Harvey, 1966).

RESULTS & DISCUSSION

Alterations in the total intramuscular fat content of the biceps femoris muscle

The rate of deposition of intramuscular fat in the biceps femoris muscle is shown in Figure 1. Fat content gradually increased during the initial stages of growth. The deposition necessary to get carcasses into the more desirable quality grades occurred rapidly during the last 2–3 months before slaughter. A similar deposition pattern was shown by Berg and Butterfield (1968).

Breed differences were apparent in that Angus calves began to deposit intramuscular fat at about 10 months, Herefords at about 14 months, and Holsteins at 16 months of age. This substantiates Lawrie's (1961) observation that beef-type cattle tend to marble at younger ages and consequently lighter weights than dairy types.

In each of the three breeds, heifers displayed a tendency for earlier fat deposition than steers. This trend was more pronounced in the Angus. This is corroborated by the earlier work of Wilson et al. (1969) and Marchello et al. (1970). Body weight difference between steers and heifers became significantly different ($P < 0.01$) at 112 days of age and remained thus for the remainder of the experiment.

The preceding considerations plus a consensus of the literature combine to provide substantial evidence for the existence of different growth curves for each sex, breed and sex-within breed. In all cases the general fat deposition patterns are similar to the variability occurring as a result of different time-weight relationships. However, other factors such as nutritional regimen and season may also contribute to observed differences.

Muscle fat classes

The data exhibit several quantitative changes in the muscle-fat class constituents during growth. Primary fat classes were phospholipids (PL), cholesterol (Chol), free fatty acids (FFA) and triglycerides (TG). In each case growth effects were definite (Fig. 2). The PL, FAA and particularly Chol significantly decreased ($P < 0.05$) in concentration, while TG underwent a substantial increase ($P < 0.05$). The classes showed a reduced rate of change in their respective concentrations with increasing age. The majority of the compositional changes occurring during growth had taken place by 224 days of age (body weight $\cong$ 182 kg). This suggests there is an increasing degree of chemical stability with growth.

As shown in Figure 2 the concentration of Chol in the muscle in the mature slaughter age animal was about 11%. Comparing this to a 28-day old calf, a decline of approximately 70% takes place during growth and fattening. This supports the earlier hypothesis of Stromer et al. (1966) that intramuscular fat contains only small amounts of Chol and that the structural components of the muscle tissue contain the higher concentrations of Chol. The TG increase reflects increases in marbling probably due to adipocyte infiltration into muscle (Moody and Cassens, 1968).

Some of the apparent growth effects observed may be related to corresponding changes in muscle fiber types (red-white-intermediate). Froberg (1967) found in the rat gastrocnemius muscle that the PL and Chol concentrations were about 50% higher in the red fibers than in the white fibers. In the present experiment (Spindler, 1972) calves exhibited a substantial increase in mean white fiber area with increased growth ($P < 0.01$). This may be one of the factors contributing to the observed decrease in PL and Chol.

Although specific trends did exist in reference to breed and sex they are not significant throughout the growth interval measured. The breed differences were limited to earlier stages of growth (< 280 days) but due to the sporadic occurrence of these differences it is difficult to place any practical importance on them. Angus calves deposited intramuscular fat faster than either Herefords or Holsteins and TG was the principle fat class responsible for the increase.

Differences in sex effects were limited to the initial biopsies (< 56 days of age) and included only Chol and TG. Heifers displayed a higher Chol ($P < 0.05$) level while the reverse was true for the TG ($P < 0.05$). Additional work is necessary to elucidate the breed and sex effect on muscle fat classes.

Subcutaneous fat classes

Although the amount of subcutaneous fat increased markedly during growth and fattening, the relative fat class concentrations experienced only minor modifications (Table 1). The PL and PFA showed a small but significant decline while the remaining classes maintained a relatively constant concentration. Hood and Allen (1973) have shown that increased subcutaneous fat deposition subsequent to 8 months of life is more a case of hypertrophy (cell enlargement) of existing adipose cells rather than hyperplasia (cell proliferation). Consequently any lipid that is principally associated with the structural components of the adipocyte (such as PL) would probably experience a decrease in its relative concentration with increased fattening. The reason for the decline in FFA is not clear and cannot be readily explained by data obtained in this study.

As in the case of the muscle fat classes the breed and sex effects on subcutaneous fat were of a minor and inconsistent nature.

Serum fat classes

Serum fat contained almost no monoglycerides (MG) or diglycerides (DG). The DG occurred occasionally but in such small amounts that they were almost *impossible* to quantitate. Because of the almost complete nonexistence of MG and the infrequent occurrence of DG, neither class was included in the serum fat class

Table 1—Least squares means of subcutaneous fat classes[a,b]

Fat class	Age in days: 56	112	168	224	280	336	392	448	SI
	Percent of total lipid								
Phospholipids	1.43^{cd}	1.76^{c}	1.48^{cd}	1.22^{def}	1.20^{de}	1.07^{ef}	1.35^{cef}	1.16^{cef}	0.93^{f}
	(0.16)	(0.25)	(0.15)	(0.24)	(0.11)	(0.11)	(0.23)	(0.35)	(0.10)
Monoglycerides	0.30	0.24	0.10	0.12	0.19	0.14	0.13	0.10	0.12
	(0.1)	(0.09)	(0.02)	(0.02)	(0.08)	(0.04)	(0.04)	(0.02)	(0.06)
Diglycerides	5.43	5.63	6.70	6.63	6.57	6.21	6.18	5.84	5.87
	(0.18)	(0.28)	(0.30)	(0.47)	(0.83)	(0.81)	(0.80)	(0.73)	(0.98)
Cholesterol	7.61	7.23	8.20	7.22	6.71	7.69	6.46	5.46	6.36
	(0.54)	(0.65)	(0.80)	(0.73)	(0.74)	(0.88)	(0.55)	(0.67)	(0.63)
Free fatty acids	6.13^{c}	5.11^{c}	4.46^{cd}	3.02^{d}	3.66^{d}	4.41^{cd}	3.43^{d}	2.84^{d}	4.11^{d}
	(0.79)	(0.48)	(0.90)	(0.45)	(0.48)	(1.10)	(0.57)	(0.71)	(0.41)
Triglycerides	77.01	77.00	77.00	79.51	79.91	77.14	78.18	81.19	78.72
	(1.30)	(1.53)	(2.13)	(2.27)	(1.48)	(2.11)	(1.98)	(1.41)	(1.40)
Cholesterol	0.78	0.94	0.58	0.51	0.36	0.43	0.44	0.80	0.53
esters	(0.28)	(0.27)	(0.17)	(0.18)	(0.11)	(14)	(0.08)	(0.34)	(0.10)

[a] Number of observations = 27.
[b] Values are least squares means and standard errors of the means.
[c,d,e,f] Means on the same line not bearing a superscript letter or means bearing a common superscript letter are not significantly ($P < 0.05$) different.

Table 2—Least squares means of serum fat classes[a,b]

Fat class	Age in days: 28	56	140	252	308	364	420	SI
	Percent of total lipid							
Phospholipids	9.24^{c}	13.76^{d}	12.29^{cd}	11.72^{cd}	13.73^{d}	14.06^{d}	11.95^{cd}	12.49^{d}
	(1.05)	(1.69)	(1.73)	(1.73)	(1.16)	(1.14)	(2.39)	(1.26)
Cholesterol	16.34	16.11	18.31	16.78	19.53	19.00	19.22	18.07
	(1.16)	(0.81)	(1.80)	(1.04)	(1.81)	(1.02)	(2.06)	(1.48)
Free fatty acids	7.61	8.94	6.96	7.51	8.46	8.69	7.89	9.50
	(0.88)	(1.11)	(1.09)	(1.18)	(1.53)	(0.74)	(1.64)	(1.33)
Triglycerides	9.39	9.56	11.80	12.57	11.81	11.89	10.82	9.85
	(1.31)	(1.68)	(1.11)	(1.35)	(2.62)	(1.57)	(1.68)	(1.65)
Cholesterol	56.69^{c}	51.92^{cd}	50.69^{cd}	52.73^{cd}	50.84^{cd}	46.67^{d}	49.85^{cd}	50.24^{cd}
esters	(2.04)	(2.63)	(3.34)	(2.59)	(3.58)	(2.45)	(4.81)	(3.10)

[a] Number of observations = 27.
[b] Values are least squares means and standard errors of the means.
[c,d] Means on the same line not bearing a superscript letter or means bearing a common superscript letter are not significantly ($P < 0.05$) different.

analysis. The remaining five classes, PL, Chol, FFA, TG and cholesterol esters (CE), all seemed to show a reasonably constant concentration which was unaffected by growth (Table 2). The slaughter samples showed close agreement with the results of Thrall and Cramer (1971). The relative concentration of serum fat classes followed the same concentration gradient throughout the growth curve, CE > Chol > PL > TG > FFA. Large changes in serum fat classes would not be expected because the presence of a functional rumen will largely standardize the type of fat presented to the intermediary metabolism.

Tissue comparisons

A general comparison of the three tissues studied reveals several basic differences in lipid composition that reflect basic cellular differences (i.e., storage, membrane and transport functions). The TG concentration maintained a consistent relative difference, subcutaneous > muscle > serum. This sequence was consistent the entire growth period. During the initial stages of growth (< 168 days) the Chol level in the muscle lipid was the highest ($P < 0.01$) followed by the serum and subcutaneous fat; however, with continued aging it declined to a point lower than that of serum ($P < 0.05$) and similar to the subcutaneous. The muscle fat PL concentration was consistently higher ($P < 0.01$) than that of subcutaneous fat. Initially muscle PL were also greater than serum ($P < 0.05$); however, with growth this trend was eventually reversed (~9 months of age). The most dramatic difference was exhibited by the relative CE concentrations. Throughout

the study the level in serum far exceeded that of muscle or subcutaneous fat depots. The remaining classes did not display any pronounced variation in their respective concentrations between tissues.

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COMPOSITIONAL AND METABOLIC GROWTH EFFECTS IN THE BOVINE
Muscle, Subcutaneous and Serum Total Fatty Acids

INTRODUCTION

IF THE BEEF industry is to produce low-fat beef in light of current demands, the product must be as acceptable as the meat consumers presently enjoy. As physical appearance and eating quality are somewhat a reflection of the fatty acid composition of the fats in meat, a study on relative changes in fat composition during growth and development would be of value to research on modification of beef composition.

Along with increases in total amount of fat, muscle fat classes exhibit significant changes with growth (Link et al., 1970a; Hecker et al., 1975). In addition, fatty acid composition of muscular and subcutaneous fats have been found to increase in unsaturation with growth (Waldman et al., 1968; Link et al., 1970b, c). The preceding studies were conducted on animals that were relatively mature (> 12 months of age). As a result, the changes in fat composition occurring during early growth remain unknown. The present investigation was designed to determine the total fatty acid composition of bovine muscle, subcutaneous and serum fat during the growth interval from 28 days of age to slaughter.

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Table 1—Total fatty acid profile of muscle lipid[a,b]

	Age in days									
	28	56	112	168	224	280	336	392	448	SI
	Average live weight, kg									
Fatty acid	33	49	88	137	182	230	275	342	390	444
C10:0	0.02^{ce} (0.01)	0.08^{cde} (0.04)	0.01^{ce} (0.01)	0.02^{c} (0.01)	0.02^{cde} (0.01)	0.03^{cde} (0.01)	0.05^{cde} (0.02)	0.05^{cde} (0.04)	0.09^{d} (0.01)	0.01^{e} (0.01)
C12:0	0.07 (0.03)	0.14 (0.05)	0.05 (0.01)	0.10 (0.04)	0.07 (0.03)	0.08 (0.02)	0.02 (0.01)	0.15 (0.09	0.10 (0.04)	0.05 (0.01)
C14:0	2.34^{cde} (0.32)	2.04^{cd} (0.26)	1.64^{c} (0.18)	2.44^{de} (0.31)	2.24^{d} (0.17)	2.9^{d} (0.15)	2.40^{d} (0.18)	2.35^{d} (0.19)	2.24^{de} (0.34)	2.77^{e} (0.15)
C14:1	0.64^{c} (0.16)	0.85^{cdf} (0.22)	0.72^{c} (0.10)	0.90^{cf} (0.15)	1.24^{def} (0.13)	1.17^{df} (0.13)	1.50^{efg} (0.14)	1.51^{efg} (0.13)	1.43^{f} (0.27)	1.65^{g} (0.10)
C15:0	0.44 (0.14)	0.30 (0.09)	0.35 (0.08)	0.21 (0.07)	0.40 (0.09	0.25 (0.09)	0.18 (0.04)	0.14 (0.05)	0.28 (0.05)	0.51 (0.06)
C16:0	26.65^{ce} (1.15)	28.04^{d} (0.92)	26.00^{cde} (1.08)	27.54^{d} (0.73)	27.75^{d} (0.68)	25.83^{e} (0.78)	26.18^{e} (1.39)	26.29^{e} (1.36)	26.81^{e} (2.23)	27.05^{cde} (0.98)
C16:1	3.98^{c} (0.44)	3.81^{c} (0.34)	4.45^{c} (0.32)	5.76^{d} (0.31)	6.39^{deg} (0.37)	7.15^{efg} (0.47)	9.48^{f} (1.10)	8.54^{f} (0.54)	9.32^{f} (0.92)	6.78^{g} (0.45)
C17:0	0.85^{cd} (0.15)	0.64^{c} (0.10)	0.87^{cd} (0.15)	0.99^{de} (0.15)	1.24^{de} (0.24)	1.10^{cde} (0.24)	1.28^{de} (0.22)	1.13^{de} (0.18)	1.18^{de} (0.09)	1.29^{e} (0.16)
Ciso18	0.45 (0.12)	0.45 (0.13)	0.31 (0.08)	0.43 (0.14)	0.48 (0.10)	0.44 (0.13)	0.23 (0.08)	0.38 (0.08)	0.40 (0.16)	0.93 (0.14)
C18:0	13.99^{ce} (1.04)	16.15^{d} (1.10)	16.79^{d} (1.33)	13.43^{c} (0.46)	11.15^{e} (0.65)	0.02^{e} (0.52)	8.28^{f} (0.62)	7.28^{fg} (0.61)	7.65^{fg} (1.90)	6.24^{g} (0.75)
C18:1	39.60^{c} (1.78)	36.13^{c} (1.18)	36.84^{c} (1.37)	33.45^{c} (0.95)	43.74^{d} (0.68)	45.20^{d} (0.94)	44.83^{d} (1.19)	48.94^{e} (1.15)	48.02^{e} (2.36)	48.07^{e} (1.48)
C18:2	10.82^{cd} (1.33)	9.98^{cd} (1.08)	10.76^{c} (0.78)	8.68^{d} (0.84)	5.12^{e} (0.73)	5.82^{e} (0.75)	4.66^{ef} (1.58)	3.31^{f} (0.33)	3.84^{f} (0.44)	5.21^{e} (0.39)
Others	1.72 (0.43)	1.99 (1.14)	0.75 (0.22)	0.86 (0.32)	0.23 (0.10)	0.64 (0.36)	0.34 (0.17)	0.30 (0.01)	0.14 (0.04)	0.32 (0.21)

[a] Expressed as % of total fatty acids and based on 27 animals
[b] Least squares means and standard errors
[c—g] Means in the same line not bearing a superscript letter or means bearing a common superscript letter are not significantly ($P < 0.05$) different.

Table 2–Muscle lipid compositional differences occurring during growth by sex and breed[a]

Age (days)	Fatty acid	Effect	Significance level	Interpretation
28	C16:0	Breed	0.01	Hol < (H = A)
56	C17:0	Breed	0.05	H > (A = Hol)
	C18:2	Sex	0.05	♀ > ♂
112	C18:2	Breed	0.01	Hol > (H = A)
168	C18:0	Sex	0.01	♀ < ♂
	C18:1	Breed	0.05	A > (H = Hol)
	C18:2	Sex	0.01	♀ > ♂
224	C16:1	Sex	0.05	♀ > ♂
	C17:0	Sex	0.05	♀ > ♂
	Ciso18	Breed	0.05	Hol > (H = A)
	C18:0	Sex	0.01	♀ < ♂
280	C18:0	Sex	0.01	♀ < ♂
	C18:1	Sex	0.01	♀ > ♂
336	C14:1	Sex	0.05	♀ > ♂
	C15:0	Sex	0.01	♀ > ♂
	C15:0	Breed	0.05	H < (A = Hol)
392	None	–	–	–
448	C15:0	Sex	0.01	♀ > ♂
	C17:0	Sex	0.01	♀ > ♂
	Ciso18	Breed	0.05	Hol > (A = H)
	C18:0	Sex	0.05	♀ > ♂
	C18:1	Breed	0.01	A < (H = Hol)
Sl[b]	C14:1	Sex	0.01	♀ > ♂
	C16:1	Breed	0.01	Hol < (A = H)
	Ciso18	Sex	0.05	♀ > ♂

[a] A = Angus, H = Hereford, Hol = Holstein
[b] Slaughter age was variable

EXPERIMENTAL

MUSCLE (biceps femoris), subcutaneous adipose tissue (tail-head) and blood (serum) samples were collected from Hereford (8 steers and 3 heifers), Angus (4 steers and 3 heifers) and Holstein (5 steers and 4 heifers) calves at regular intervals from 28 days of age to slaughter. Animal management and sample collection are described in detail by Hecker et al. (1975).

Fatty acid concentrations were determined by gas-liquid chromatographic (GLC) separation of methyl esters. The esterification technique was based on the transesterification method described by Cramer and Marchello (1964).

The data analysis was conducted using the least squares procedure for analysis of data with unequal subclass numbers (Harvey, 1966).

RESULTS & DISCUSSION

Muscle fatty acid profiles, growth effects

Twelve specific fatty acids were identified on the gas-liquid chromatograms. The remaining peaks since they, in toto, represented only a very small proportion (usually < 1.0%) of the total acids were pooled into one group for statistical analysis. Preliminary characterizations of these peaks by comparison to retention times of fatty acid standards obtained from the Hormel Institute indicated them to be C8:0, C9:0, C18:3 and C20:4.

Analysis of percentage composition indicated that significant changes occurred in muscle fatty acids during growth, particularly during the early stages of development (Table 1). Of the 12 fatty acids identified only C12:0, C15:0 and Ciso18 failed to exhibit a significant change at several points along the growth curve. The most dramatic changes were displayed by the increases in C14:0, C14:1, C16:1 and C18:1 and the decreases in C18:0 and C18:2. The changes observed in the remaining three acids, C10:0, C16:0 and C17:0, were of a minor and sporadic nature.

As the animals grew, there was an apparent increase in monounsaturation and a corresponding decrease in saturation. The majority of this change was accounted for by the inverse relationship between C18:0 and C18:1. Similar trends in the change in saturation have been reported by Lawrie (1961) and Link et al. (1970b). C14:1, C16:1 and C18:1 all increased but C18:2 decreased. This corroborates the findings of Waldman et al. (1968) and Link et al. (1970b). Increased activity by the fatty acid desaturase scheme with age may be one of the reasons for the decrease in saturation with growth. Physiological maturity could be the trigger that induces increased desaturation. The reason for the decline in the major polyunsaturate, C18:2, while the remaining unsaturated fatty acids increased may be a result of less of the total body fat coming from dietary and rumen microorganismal lipid. As animals mature they deposit increasing amounts of fat in the body tissues but dietary lipid remains constant at slightly less than 5% of the ration. However, other causes of decreased polyunsaturates should not be overlooked. For example, the decreases may be related to the relative change in concentration among the fat classes. Terrell and Bray (1969) reported that phospholipids have a high affinity for acids more unsaturated than C18:1. The unsaturated acids with less than 18 carbons are primarily associated with the neutral lipids. Since an earlier report from this laboratory (Hecker et al., 1975) has shown the concentration of the phospholipids in these animals (as a percent of total fat) to decrease with growth, any acid that shows a high specificity for phospholipids would also be expected to decrease proportionally via simple dilution resulting from the increase in neutral lipid fatty acids. The same form of reasoning can be applied to the increase of C14:1, C16:1 and C18:1. They possess a high affinity for neutral lipids and since the neutral lipids increases with continued growth (Hecker et al., 1975; Link et al., 1970a) the relative amounts of these fatty acids would be expected to increase. Changes in fat classes may be an important factor in the apparent alteration in specific fatty acid concentrations, but the increased monounsaturation is of such magnitude as to suggest that the activity of a desaturase system is likely increasing with growth.

Muscle fatty acids, sex effects

The earlier reports of Terrell et al. (1969) and Waldman et al. (1968) showed steers to possess greater amounts of C14:0, C16:0 and C18:0 in the muscle fat than heifers of the same weight. However a comparison on a constant weight basis results in the slower gaining heifers being much older than the steers which would make the interpretation of results ambiguous. The current data present a very random and inconsistent picture of the sex differences during growth (Table 2). A given fatty acid such as C18:2, may show a sex effect at 56 days of age but fails to retain this difference at later ages. Whether these occasional differences are real or artifactual is difficult to determine. It should be noted however that in most cases where differences do exist involving unsaturated fatty acids, heifers tend to exhibit a greater concentration than steers.

The present investigation is *more in* agreement with Link et al. (1970b) and Hood and Allen (1971) who suggest that sex does not contribute a great deal to

Table 3—Total fatty acid profile of subcutaneous lipid[a,b]

Fatty acid	Age in days: 56	112	168	224	280	336	392	448	SI
	Average live weight, kg: 49	88	137	182	230	275	342	390	444
C10:0	0.20^{cd} (0.11)	0.07^{c} (0.03)	0.07^{cd} (0.03)	0.04^{c} (0.01)	0.04^{c} (0.01)	0.01^{d} (0.00)	0.01^{d} (0.01)	0.05^{cd} (0.03)	0.00^{d} (0.00)
C12:0	0.64^{c} (0.08)	0.17^{d} (0.02)	0.16^{d} (0.04)	0.06^{e} (0.01)	0.12^{de} (0.03)	0.03^{e} (0.01)	0.03^{e} (0.02)	0.06^{e} (0.03)	0.04^{e} (0.02)
C14:0	6.55^{c} (0.63)	3.81^{d} (0.19)	3.79^{e} (0.31)	3.67^{d} (0.46)	3.27^{de} (0.17)	3.22^{de} (0.17)	3.38^{de} (0.12)	3.57^{de} (0.44)	3.29^{e} (0.13)
C14:1	1.65^{cd} (0.24)	1.23^{c} (0.12)	1.19^{c} (0.11)	1.52^{c} (0.20)	1.93^{d} (0.14)	1.84^{d} (0.14)	2.01^{d} (0.13)	2.51^{de} (0.27)	2.66^{e} (0.28)
C15:0	0.54^{cf} (0.13)	0.43^{cf} (0.09)	0.43^{cf} (0.07)	0.35^{cd} (0.07)	0.23^{de} (0.06)	0.17^{e} (0.05)	0.20^{e} (0.05)	0.15^{e} (0.12)	0.53^{f} (0.12)
C16:0	25.72^{cde} (1.22)	25.01^{cd} (0.82)	24.10^{c} (0.74)	24.23^{c} (0.50)	26.98^{de} (1.05)	26.75^{e} (0.64)	25.36^{cd} (0.52)	26.21^{ed} (2.04)	27.76^{e} (1.22)
C16:1	5.46^{c} (0.19)	5.26^{c} (0.33)	6.09^{cdf} (0.46)	6.59^{df} (0.37)	8.20^{e} (0.44)	8.57^{e} (0.65)	7.72^{ef} (0.37)	7.04^{f} (0.71)	6.34^{ef} (1.78)
C17:0	1.59^{c} (0.17)	1.68^{c} (0.15)	1.96^{c} (0.15)	1.81^{c} (0.14)	1.12^{d} (0.15)	1.37^{d} (0.17)	1.41^{d} (0.13)	1.16^{d} (0.23)	1.17^{d} (0.20)
Ciso18	0.18^{c} (0.06)	0.39^{cd} (0.12)	0.40^{cd} (0.12)	0.43^{d} (0.07)	0.49^{d} (0.17)	0.29^{cd} (0.09)	0.49^{d} (0.11)	0.30^{de} (0.03)	1.02^{e} (0.26)
C18:0	15.83^{c} (1.22)	19.20^{d} (1.15)	19.57^{d} (0.94)	17.07^{c} (0.94)	11.02^{e} (0.75)	10.48^{e} (0.84)	10.18^{e} (0.52)	7.32^{e} (2.20)	4.05^{f} (1.05)
C18:1	37.39^{c} (1.04)	38.37^{c} (0.81)	38.29^{cd} (0.82)	40.93^{d} (1.13)	44.12^{e} (1.01)	44.72^{e} (0.98)	46.55^{e} (0.54)	48.03^{e} (3.53)	49.61^{f} (1.10)
C18:2	4.55^{c} (0.44)	4.54^{c} (0.39)	3.92^{ce} (0.53)	2.95^{d} (0.24)	2.94^{de} (0.34)	2.73^{de} (0.36)	2.55^{d} (0.31)	3.28^{de} (0.41)	3.42^{e} (0.41)
Others	0.01 (0.01)	0.50 (0.38)	0.29 (0.14)	0.03 (0.01)	0.09 (0.04)	0.03 (0.01)	0.02 (0.01)	0.02 (0.01)	0.00 (0.00)

[a] Expressed as % of total fatty acids and based on 27 animals
[b] Least squares means and standard errors
[c—g] Means in same line not bearing a superscript letter or means bearing a common superscript letter are not significantly ($P < 0.05$) different.

Table 4—Subcutaneous lipid compositional differences occurring during growth by sex and breed[a]

Age (days)	Fatty acid	Effect	Significance level	Interpretation
56	C14:0	Breed	0.05	A >(H = Hol)
	C16:1	Breed	0.01	A < (H = Hol)
112	C14:0	Breed	0.05	Hol < (A = H)
	C16:0	Breed	0.01	Hol < (A = H)
	C17:0	Breed	0.01	Hol > (A = H)
168	C14:0	Sex	0.05	♀ < ♂
224	None	—	—	—
280	C14:1	Sex	0.01	♀ > ♂
	C16:1	Sex	0.05	♀ > ♂
	C18:0	Sex	0.01	♀ < ♂
336	None	—	—	—
392	C14:0	Sex	0.01	♀ < ♂
	C14:1	Breed	0.01	A > (H = Hol)
	C15:0	Breed	0.01	Hol < A < H
	C16:0	Sex	0.01	♀ < ♂
	C18:1	Sex	0.05	♀ > ♂
	C18:1	Breed	0.01	A < (H = Hol)
448	C14:0	Breed	0.01	A > (H = Hol)
	C14:1	Breed	0.01	A > (H = Hol)
	C18:2	Sex	0.05	♀ > ♂
SI[b]	None	—	—	—

[a] A = Angus, H = Hereford, Hol = Holstein
[b] Slaughter age was variable.

relative compositional differences in intramuscular fatty acids.

The sex effect can apparently be expected to result in more definite compositional differences when intact males are compared to steers and heifers than when steers and heifers are compared (Hood and Allen, 1971; Gillis et al., 1973).

Muscle fatty acids, breed effects

Thrall and Cramer (1971) after a comprehensive study of bovine fat composition in 373 animals concluded that in general breed differences (at slaughter) did not appear to be significant. In contrast, Gillis et al. (1973) reported significant differences by comparing Simmental and Limousin crossbred combinations. A comparison of the Hereford and Angus combinations in the Gillis study revealed only C16:1 to be significant and that difference was very minor (Angus = 4.8% vs. Herefords = 4.7%). The present study measured breed effects from 28 days of age to slaughter, and failed to show any consistent difference. Table 2 lists the ages and fatty acids involved in the sporadic occurrence of the significant breed effects.

Subcutaneous fatty acids

The amount of subcutaneous fat increases markedly during growth and fattening. The composition of this fat is also subject to change during the growing period as indicated by Waldman et al. (1968) who found that the percent of unsaturated fatty acids in subcutaneous fat increased as the animals increased in weight and fatness. In contrast Hornstein et al. (1967) reported that triglyceride fatty acid composition was comparatively unaffected by different ages and diets in cattle. The present investigation indicated that the degree of unsaturation increased substantially with growth ($\sim$ 49% at 28 days of age and $\sim$ 62% at slaughter) (Table 3). The saturated acids showing the greatest decline were C12:0, C14:0 and C18:0. C14:1, C16:1 and C18:1 were responsible for the majority of the increase in unsaturation. Of the remaining acids C10:0, C15:0, C17:0 and C18:2 decreased, Ciso18 increased and C16:0 remained essentially unchanged. As in muscle, the composition of subcutaneous lipids are probably a reflection of increased fatty acid synthesis and desaturation and less of an effect of dietary lipid.

It was evident from the data that the rate of change tended to slow with increasing age. In each case the most rapid rate of change was observed during the initial 12–14 months of growth. This trend was similar to the growth effect seen in intramuscular fatty acids and fat classes (Hecker et al., 1975).

The sex and breed effects were very limited (Table 4). The results were sporadic and did not demonstrate any consistent significant influence.

Table 5–Total fatty acid profile of serum lipids[a,b]

	Age in days							
	28	56	140	252	308[c]	364	420	SI
	Average live weight, kg							
Fatty acid	33	49	112	206	252	308	366	
C10:0	0.13^{d} (0.03)	0.12^{d} (0.03)	0.08^{de} (0.03)	0.05^{e} (0.02)	0.11^{de} (0.05)	0.13^{de} (0.03)	0.10^{de} (0.03)	0.09^{d} (0.02)
C12:0	0.19^{d} (0.03)	0.39^{d} (0.11)	0.38^{d} (0.12)	0.09^{e} (0.04)	0.22^{de} (0.22)	0.15^{e} (0.04)	0.19^{e} (0.06)	0.20^{de} (0.11)
C14:0	2.74^{d} (0.20)	2.27^{e} (0.14)	1.73^{e} (0.16)	1.35^{g} (0.13)	1.58^{fg} (0.40)	1.74^{fg} (0.20)	1.85^{fg} (0.30)	1.59^{g} (0.16)
C14:1	0.60^{d} (0.07)	0.89^{e} (0.10)	1.07^{e} (0.17)	1.16^{e} (0.17)	1.66^{e} (0.28)	1.01^{e} (0.12)	0.68^{d} (0.10)	0.89^{ce} (0.19)
C15:0	1.46 (0.20)	1.43 (0.17)	1.55 (0.27)	1.34 (0.13)	1.43 (0.15)	1.86 (0.75)	1.56 (0.24)	1.20 (0.13)
C16:0	27.16^{d} (1.34)	23.26^{e} (0.95)	21.32^{ef} (0.91)	19.78^{f} (0.73)	22.02^{ef} (1.23)	19.09^{f} (1.04)	19.97^{ef} (1.23)	20.40^{ef} (0.94)
C16:1	3.54^{de} (0.34)	3.38^{de} (0.03)	3.79^{ge} (0.47)	4.25^{d} (0.48)	4.81^{d} (0.57)	3.27^{ef} (0.36)	2.77^{f} (0.34)	3.58^{de} (0.40)
C17:0	2.12 (0.27)	1.48 (0.26)	2.31 (0.39)	2.25 (0.29)	2.11 (0.53)	1.84 (0.21)	2.12 (0.44)	1.56 (0.21)
Ciso18	0.20^{d} (0.08)	0.38^{de} (0.09)	0.73^{e} (0.21)	0.42^{e} (0.07)	0.65^{e} (0.24)	0.65^{e} (0.13)	0.65^{e} (0.38)	0.77^{e} (0.27)
C18:0	24.42^{d} (1.75)	24.28^{d} (1.78)	28.44^{e} (1.94)	31.97^{e} (2.16)	27.95^{de} (3.14)	28.42^{de} (1.94)	23.29^{de} (2.27)	29.95^{e} (1.97)
C18:1	24.74^{d} (1.65)	23.85^{de} (1.42)	21.25^{e} (1.38)	24.63^{de} (1.62)	21.46^{de} (2.55)	25.92^{de} (2.84)	24.97^{de} (2.85)	24.05^{de} (1.89)
C18:2	10.48^{d} (2.07)	15.12^{e} (2.30)	15.10^{e} (2.10)	11.12^{de} (1.56)	16.14^{e} (2.74)	13.92^{de} (2.05)	19.96^{e} (3.44)	13.08^{de} (2.08)
Others	1.77 (0.43)	1.77 (0.54)	1.40 (0.38)	1.17 (0.20)	2.53 (0.64)	1.67 (0.53)	2.08 (0.86)	2.22 (0.60)

[a] Expressed as % of total fatty acids and based on 27 animals

[b] Least squares means and standard errors

[c] Angus calves are not represented.

[d–g] Means in the same line not bearing a superscript letter or means bearing a common superscript letter are not significantly ($P < 0.05$) different.

Serum fatty acids

The importance of serum lipids to carcass fat deposition has not been definitely determined. Dryden et al. (1973) suggested that the fatty acid composition of certain subcutaneous depots may be a reflection of the change in fatty acid composition seen by the serum free fatty acid and glyceride fractions as affected by dietary regimes or time. Consequently an awareness of the composition of the fats present in the serum is germane to attempts to control the composition of body fats.

The serum fatty acids are summarized in Table 5. The most consistent changes are shown by the decrease in C14:0 and C16:0. The remaining fatty acids all displayed considerable variability. Thrall and Cramer (1971) and Marchello et al. (1971) reported that even under controlled conditions blood lipids tended to be highly variable.

Table 6 shows the statistically significant sex and breed effects in fatty acid composition of serum lipids at various stages of growth. There did not seem to be any consistent pattern which indicates that the composition of serum lipids is not influenced by sex or breed.

Tissue comparison

A general comparison showed serum lipids to be more saturated than either muscle or subcutaneous fats (Fig. 1). This was true for all biopsy periods. A striking exception to this was the high relative concentration of C18:2 in the serum fat. This difference was probably the result of a much higher level of cholesterol esters in serum (Hecker et al., 1975). Hernandez and Chaikoff (1957) reported that unsaturated acids were preferentially esterified to cholesterol. Thrall and Cramer (1971) reported the C18:2 concentration in cholesterol ester fatty acids to be as high as 80%. Consequently a high level of cholesterol esters in the serum fat would result in a higher concentration of C18:2.

Initially, intramuscular lipids displayed a greater degree of unsaturation than subcutaneous lipids. However, with continued growth and fattening, the trend was reversed. In both cases the overall trend was for increased unsaturation while in the serum fat the ratio of unsaturated to saturated fatty acids was relatively constant.

The higher level of unsaturation observed in muscle lipids during the initial 14 months of growth was contrary to the earlier reports of Link et al. (1970b, c) and Clemens et al. (1973) who found the subcutaneous adipose tissue to possess a higher level of unsaturated fatty acids at least in animals over 1 yr of age. A satisfactory explanation for these contradictory results is not readily apparent from the present data. However, it must be kept in mind that these animals were maintained in an environmentally controlled facility until they were approximately 12 months old, which, according to Cramer and Marchello (1964) and Link et al. (1970b, c) would eliminate compositional changes due to temperature effects. Also, the possibility should not be ignored that the biopsy trauma could cause physiological changes which would result in abnormal lipid patterns. In addition, the tail-head region may present a somewhat different compositional picture than other body locations. Terrell et al. (1967) found the unsaturated/saturated ratio for subcutaneous lipids to range from 1.18–1.50 among several carcass locations. Gillis et al. (1973) using the biceps femoris and a corresponding subcutaneous fat sample, found no difference in the overall unsaturated/saturated ratio between the two tissues. Therefore the results noted in this experiment may simply be the consequence of a location difference.

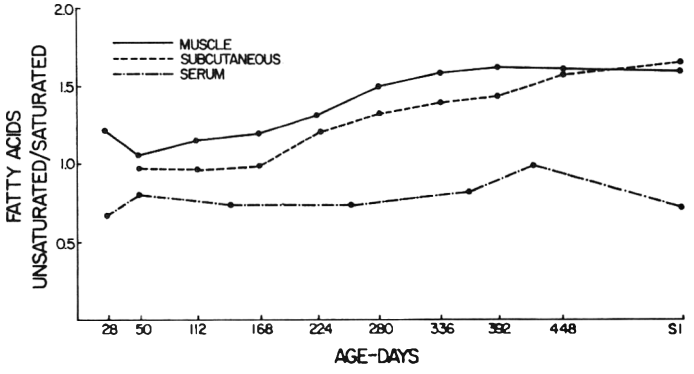

Fig. 1—Ratio of unsaturated fatty acids:saturated fatty acids in muscle, subcutaneous and serum fat.

Table 6—Serum lipid compositional differences occurring during growth by sex and breed[a,b]

Age (days)	Fatty acid	Effect	Significance level	Interpretation
28	Total fat (mg%)	Breed	0.05	H < (A = Hol)
	C16:1	Breed	0.05	A < (H = Hol)
56	C14:0	Breed	0.05	Hol < (A = H)
	C14:1	Breed	0.01	A > (H = Hol)
	C14:1	Sex	0.01	♀ > ♂
	C16:1	Breed	0.05	A < (H = Hol)
	C17:0	Sex	0.05	♀ < ♂
140	None	None	—	—
252	None	—	—	—
308	C18:2	Breed	0.05	H > Hol
364	C16:0	Sex	0.05	♀ < ♂
420	C14:1	Sex	0.05	♀ > ♂
	C14:1	Breed	0.01	A > (H = Hol)
	C16:1	Sex	0.05	♀ > ♂
	C18:0	Sex	0.01	♀ < ♂
SI[c]	None	—	—	—

[a] A = Angus, H = Hereford, Hol = Holstein
[b] Angus samples not available at 308 days of age
[c] Slaughter age was variable.

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CHANGES IN TENDERNESS AND COLLAGEN OF BEEF SEMITENDINOSUS MUSCLE HEATED AT TWO RATES

INTRODUCTION

THE EFFECT of heating on the tenderness of meat is a complex problem. Although tenderness related changes occurring in meat during heating have been studied extensively many questions remain unanswered. Several investigators including Paul (1963), Hamm (1966) and Draudt (1972) have theorized that heat related changes in meat tenderness result from two opposing effects. Changes in connective tissue have a tenderizing effect while hardening of the myofibrillar proteins has a toughening effect.

Roasting of beef at very low oven temperatures (66–121°C) for long periods of time produced more tender meat than roasting at a higher temperature (149–163°C) for a shorter time (Bramblett et al., 1959; Bramblett and Vail, 1964; Bayne et al., 1969). Paul (1963) and others suggested that slower rates of heat penetration resulting in prolonged periods of time in the 57–60°C range may promote greater degradation or softening of connective tissue without extensive hardening of muscle fibers.

In studies on changes in connective tissue, alkali insoluble collagen decreased during heating (Ritchey and Cover, 1962; Ritchey et al., 1963; McClain et al., 1965; Bayne et al., 1971). However, Bayne et al. (1971) did not find that the amount of residual collagen changed with different heating rates.

Increased tenderness of meat heated at very low temperatures for long periods of time may be attributable partially to the action of enzymes. Laakkonen et al. (1970b) reported the presence of proteolytic and collagenolytic-like activity in meat heated to 60°C at slow rates.

The present investigation focused on changes in the connective tissue (CT) component of semitendinosus cores heated in a water bath at rates comparable to oven roasting of 2 kg top rounds at 93°C and at 149°C. These changes were related to changes in tenderness as measured by Warner Bratzler shear. Samples were heated to four end points, 40, 50, 60 and 70°C to evaluate changes in CT heated in the intact muscle tissue and in CT isolated from muscle prior to heating. Solubilization of hydroxyproline was measured in the intact tissue and more specific changes in the collagen molecule were studied in the isolated CT. The occurrence of proteolytic activity in the muscle and in the drip lost during heating also was investigated.

EXPERIMENTAL

Source and preparation of samples

One semitendinosus (ST) muscle from each of four Hereford × Charolais-Hereford (H × CH) and three Hereford × Charolais (H × C) heifers was secured from the Animal Science Department. Age of the animals ranged from 428–493 days. Mean USDA quality grade for each breed group was high good (Winfree, 1973). The muscles were excised after 8–10 days aging at 0.6°C and placed in freezer storage for 10–11 months.

Each frozen muscle was cut into sections 57 mm long across the fibers. Epimysial connective tissue and adhering fat were removed while the muscle sections were allowed to thaw slightly to facilitate removal of 16 cores, 2.5 cm in diameter. Cores were taken parallel to the muscle fibers, placed in 50 ml pyrex test tubes and randomly assigned to heating rate-end point temperature treatments. Two additional cores were taken and assigned to thermocouples for monitoring heating rates.

The remaining tissue was ground once through a plate with 10 mm holes and mixed well. Samples were taken for pH determinations and isolation of crude connective tissue (CCT) by the method of Field (1970).

Heating

Tubes containing cores of meat and tubes containing 1.0–1.8g of CCT in 35 ml of 0.1M potassium phosphate buffer, pH equivalent to that of the raw muscle (pH 5.59–5.84) were placed in a shaker water bath containing chilled water (6–12°C). The temperature control of the water bath was adjusted periodically in order to approximate the heat penetration curves (Fig. 1) obtained in previous work. The heating rates simulated oven roasting of 2 kg top round roasts at 93°C for approximately 9½ hr (slow heating) and at 149°C for approximately 2½ hr (fast heating). Two tubes containing cores and two containing CCT-buffer samples were removed when corresponding samples containing copper constantan thermocouples reached temperatures of 40, 50, 60 and 70°C. Tubes were cooled in an ice bath for 15 min.

Preparation for evaluation

Cores were removed from tubes, adhering drip was removed and cores were refrigerated. The drip was homogenized at low speed in a Virtis homogenizer for 30 sec and sampled for further analysis.

The buffer from each CCT-buffer tube was decanted into a mixing cylinder. A glass stirring rod was used to press excess buffer from the CCT residue. The residue was weighed and subsampled for the determination of hydroxyproline and guanidine hydrochloride soluble intramuscular collagen (GSIC). The remaining buffer was added to that in the mixing cylinder and made to a volume of 80 ml.

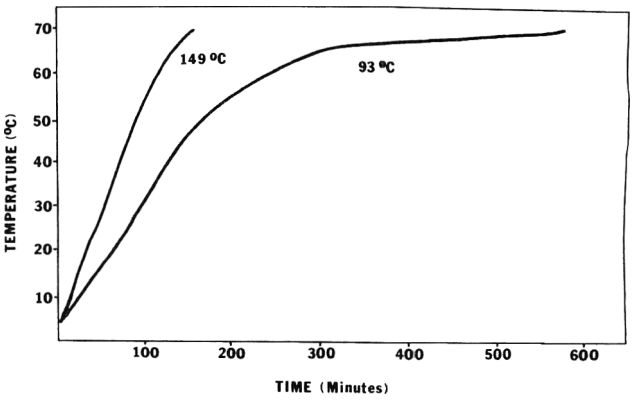

Fig. 1—Time-temperature curves from oven roasting of top round roasts at 93° C and at 149° C.

[1] Deceased, June 5, 1974.

Shear values

Following overnight refrigeration each core was sheared three times with a Warner Bratzler shear. Sheared cores were ground once through a plate with 4 mm holes, mixed well and apportioned for analysis.

Moisture-fat determinations

Duplicate 3–5g samples of ground muscle were dried in a vacuum oven at 60°C for 16 hr, weighed and extracted with petroleum ether (b.p. 37.4–50.0°C) for 6 hr on a Goldfisch Fat Extraction Apparatus. The residue was redried, weighed and percentage of nonfat dry weight (% NFDW) calculated.

Hydroxyproline determinations

Woessner's (1961) Methods I and II were used for hydroxyproline determinations. Method I was used for buffer, CCT and GSIC samples and Method II was used for muscle and drip samples.

Solubilization of hydroxproline in cores

A modification of the method described by Paul et al. (1973) was used to determine the amount of hydroxyproline solubilized during heating of the cores. The term hydroxyproline solubilized rather than collagen solubilized is used since correction of hydroxyproline values for elastin content was not made.

5g of muscle tissue from each treatment were homogenized with 50 ml distilled water (40°C) in a Waring Blendor for 2 min. The homogenate was centrifuged at 4,600 × G for 15 min. The supernatant was decanted through cheesecloth, made to volume (80 ml) and analyzed for hydroxyproline content. The percentage of solubilized hydroxyproline was calculated.

Solubilization of hydroxyproline in buffer system

Hydroxyproline content of the CCT and buffer was determined as above. Percentage of hydroxyproline solubilized during heating was calculated.

Solubility of heated CCT in guanidine hydrochloride

Guanidine hydrochloride soluble intramuscular collagen (GSIC) was extracted from 1.9–2.4g of CCT from each treatment. The CCT was extracted twice with 20 ml portions of 4M guanidine hydrochloride for 24 hr in the cold (2–3°C). The second extraction was preceded by centrifugation at 2,000 × G at 2–3°C for 10 min. The combined supernatants were dialyzed against several changes of cold demineralized water for 7 days in the cold to precipitate the GSIC. The GSIC suspension was centrifuged at 2–3°C for 20 min, decanted and the precipitate completely solubilized in 50 ml of 0.5M acetic acid. The percentage yield of GSIC was calculated from hydroxyproline values for GSIC-acetic acid mixtures and CCT samples.

Testing for proteolytic enzyme activity

A modification of the methods described by Laakkonen et al. (1970a) and Kronman et al. (1960) was used for the isolation of water soluble fractions. 16g raw or heated ground tissue or one-half of the drip in 50 ml cold demineralized water was stirred for 1 hr on a cold plate (2–3°C). Duplicate pH measurements were taken on each muscle sample immediately after stirring. Each mixture was centrifuged at 25,500 × G for 1 hr at 2–3°C. The supernatant was decanted through cheese cloth and made to a volume of 64 or 75 ml for tissue and drip samples respectively.

Azocoll, an insoluble powdered cowhide-azo dye complex, was used as a substrate to test for proteolytic activity. The azo dye is released from the collagen proparation by the action of proteolytic enzymes, the rates of release being indicative of the degree of proteolytic activity of the test material (Laakkonen et al., 1970b).

Six 10 ml portions of each water soluble fraction were added to 35 mg of Azocoll. Three tubes were incubated at 37°C and three were kept at 0°C in the ice bath for 15 min. For each replication, blanks containing 35 mg of Azocoll and 10 ml of 0.1M phosphate buffer, pH equivalent to the raw sample, also were prepared and incubated at 0 and 37°C.

Following incubation, activity was terminated by filtration of the samples and blanks through Whatman No. 2 filter paper. Absorbance (Abs) of each filtrate was measured at 520 nm.

Enzyme activity values (EAV) for the core and drip samples were calculated using the following equations:

$$EAV_{core} = \frac{\left(\text{Abs of 37°C sample}\right) - \left(\text{Abs of 37°C blank}\right) - \left(\text{Abs of 0°C sample}\right) + \left(\text{Abs of 0°C blank}\right)}{\%\ \text{NFDW} \times 10^{-2}}$$

$$EAV_{drip} = 2\left[\left(\text{Abs of 37°C sample}\right) - \left(\text{Abs of 37°C blank}\right) - \left(\text{Abs of 0°C sample}\right) + \left(\text{Abs of 0°C blank}\right)\right]$$

The absorbance of each core sample was divided by % NFDW to correct for differences in moisture content.

No attempt was made to convert absorbance values to activity units of a specific enzyme using curves supplied by the manufacturer.

RESULTS & DISCUSSION

Heating rates

Mean times required to reach each end point in the cores and in the CCT-buffer systems are presented in Table 1. Mean times required to reach 70°C were 3½ times as long with the slower rate of heating as with the fast rate.

Shear values

A summary of the results of analysis of variance of shear values is given in Ta-

Table 1—Characteristics of semitendinosus muscle and connective tissue samples heated at two rates to four end points

	Treatment							
	Slow heating rate				Fast heating rate			
	40°C	50°C	60°C	70°C	40°C	50°C	60°C	70°C
Time to reach end point, min								
a. cores	134	176	247	575	82	105	127	169
b. CCT-buffer system	123	165	212	546	75	93	115	150
Shear value, kg	19.0	17.7	6.9	5.2	19.3	17.8	7.4	7.4
Hydroxyproline solubilized in cores (%)	1.95[a]	2.54[a]	5.73[b]	13.43[c]	1.28[a]	2.84[a]	5.52[b]	9.03[d]
Proteolytic enzyme activity values (Absorbance)								
a. cores	0.073	0.071	0.097	0.060	0.106	0.087	0.098	0.038
b. drip	0.025	0.017	0.013	0.007	0.020	0.008	0.002	0.005
pH of cores	5.59	5.69	5.77	5.84	5.62	5.70	5.76	5.81
Hydroxyproline solubilized in CCT-buffer system (%)	0.00	0.05	2.80[a]	12.16[b]	0.00	0.00	1.69[a]	5.16[c]
GSIC yield (%)	2.70	1.95	2.20	0.89	2.60	2.62	2.47	1.48

[a,b,c,d] Means in the same row with like superscripts do not differ significantly ($P < 0.01$; Student-Newman-Keuls Test).

ble 2. Cores from H x C muscles were less tender ($P < 0.05$) than the cores from H x CH muscles. Mean values for the two breed groups were 13.4 ± 1.3 and 11.9 ± 1.0 kg respectively.

Cores heated at the fast rate were less tender ($P < 0.05$) than those heated at the slow rate. Respective mean shear values for the two rates were 13.0 ± 1.1 and 12.1 ± 1.2 kg. These findings are in agreement with those of earlier workers (Bramblett et al., 1959; Bramblett and Vail, 1964; Bayne et al., 1969).

Shear values differed with respect to end point temperatures ($P < 0.001$). The change is illustrated by the end point means (Fig. 2). A small but significant decrease in shear values occurred as cores were heated from 40–50°C. A greater decrease occurred in the 50–60°C interval. Heating from 60–70°C did not significantly reduce shear values.

Tenderness of ST muscle was associated with end point in several earlier studies. Laakkonen et al. (1970a) found that the major decrease in shear values of ST muscle heated at a slow rate occurred as the meat warmed from 50–60°C. On the other hand, Paul et al. (1973) reported that end point did not influence tenderness of ST cores. A decrease in shears with heating in the range 56–59°C as a result of collagen shrinkage was reported (Machlik and Draudt, 1963).

The effect of heating rate was independent of the effect of end point temperature. However, the treatment means (Table 1) suggest a trend toward increased tenderness between 60 and 70°C at the slower rate of heating.

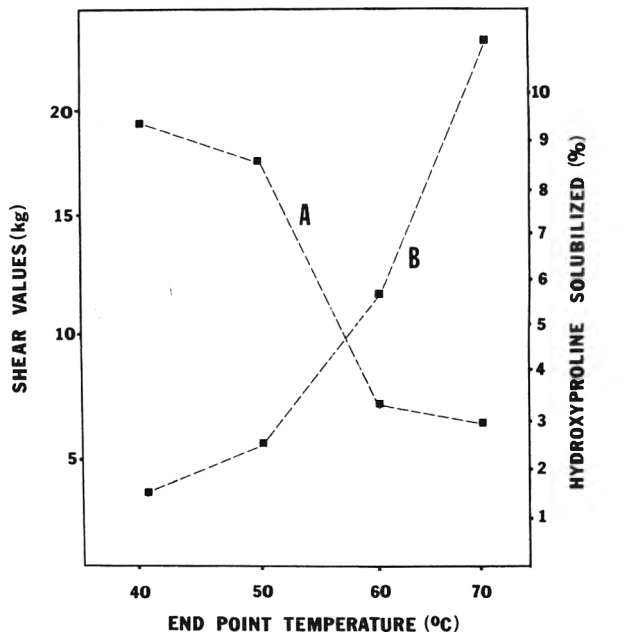

Fig. 2—The effect of end point temperature on shear values (A) and percentage of hydroxyproline solubilized (B).

Solubilization of hydroxyproline in heated ST cores

Solubilization of hydroxyproline values are given in Table 1. Heating rate had an influence on solubilization in cores. At the slower rate without respect to end point, 5.92 ± 0.96% of the hydroxyproline was solublized. This value was greater ($P < 0.01$) than the 4.67 ± 0.70% solublized with the fast rate of heating.

As end point increased, solubilization of hydroxyproline increased ($P < 0.01$) as shown in Figure 2. An increased solubilization of collagen in ST with increasing internal temperature was reported by Paul et al. (1973).

Relationship between hydroxyproline solubilization and shear values

There was a significant relationship be-

Table 2—F values from analysis of variance of shear values, solubilization of hydroxyproline, proteolytic enzyme activity, peptide lengths and GSIC yield.

Source	Degrees of freedom	F Values				
		Shears	% Hydroxyproline solubilized in cores	Enzyme activity		% Yield GSIC
				Cores	Drip	
Rate	1	7.7**	8.94**	<1	5.27*	6.07*
End point	3	438.1***	106.45***	4.20*	7.00***	19.03***
Linear	1	1133.9***	289.76***	4.93**	18.91***	43.63***
Quadratic	1	1.1	29.31**	3.33	2.18	7.27*
Cubic	1	179.3***	<1	4.33**	0	6.23*
Rate X end point	3	2.1	6.56**	1.20	<1	1.43
Breed	1	12.3*	1.76	4.04	<1	1.29
Rate X breed	1	1.5	3.79	<1	<1	<1
End point X breed	3	1.8	<1	<1	<1	<1
Rate X end point X breed	3	<1	<1		<1	<1
Animal/breed[a]	5					
Treatment X animal/breed[b]	35					

[a] Error term for breed
[b] Error term for all sources except breed
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

tween shear values and percentage of hydroxyproline solubilized during heating of cores. As percentage of hydroxyproline solubilized increased, shear values decreased ($r = -0.704$; $P < 0.01$). The relationship is illustrated in Figure 2. There appear to be limitations to this relationship, however. A small but significant ($P < 0.01$) decrease in shear value from 40–50°C was not paralleled by a significant increase in hydroxyproline solubilization at the slow or fast rate. A significant increase in solubilization of hydroxyproline from 50–60°C was accompanied by a significant decrease in shear values.

The significant difference in solubilized hydroxyproline between the slow and fast 70°C cores (Table 1) did not result in a significant difference in shear value between the two samples. The increased solubilization in the slow 70°C cores might have been overshadowed by other factors important in the determination of tenderness such as hardening of the myofibrillar proteins during the long period of heating from 60 to 70°C. After comparing shear values and penetration data of ST strips, Paul et al. (1973) concluded that CT breakdown was less important than muscle fiber coagulation in control of tenderness changes in the strips heated to 82°C. Correlation coefficients between percentage of solubilized collagen and shear values were not significant. Differences in the end point temperatures studied might be partially responsible for the differences in the results of the Paul et al. (1973) study and the present investigation. Draudt (1972) implied that hardening of the myofibrillar proteins occurred between 60 and 74°C. Heating above 70°C as in the study of Paul et al. (1973) might have resulted in greater hardening than was seen in the present study.

Limitations in the relationship between tenderness and labile collagen were recognized by Field et al. (1970). At low shear values, increased labile collagen has little effect on tenderness. The effects of heating rate and end point temperatures on shear values cannot be completely explained in terms of percentage of hydroxyproline solubilized.

Proteolytic enzyme activity

Proteolytic enzyme activity was exhibited in cores and drip from all treatments (Table 1). End point had a significant ($P < 0.05$) influence on the level of enzyme activity (EAV) in water soluble extracts of heated cores (Table 2). A curvilinear trend was found and end point means are illustrated in Figure 3. Rate of heating did not affect the level of enzyme activity.

A different pattern of proteolytic activity was exhibited by the drip samples. A linear ($P < 0.001$) decrease (Fig. 3, Curve B) in activity was shown. Drip lost from slowly heated cores exhibited greater ($P < 0.05$) proteolytic activity than the drip from cores heated at the faster rate. Respective mean activity values were 0.014 ± 0.002 and 0.009 ± 0.003.

From these data it is evident that general proteolytic enzymes could be active during heating of meat. Landmann (1963) noted that the optimal pH for a proteinase system isolated from beef muscle was in the range, 5–6. All muscle samples in this study were within this range (Table 1). Although Bodwell and Pearson (1964) found that the sarcoplasmic proteins are the major substrate for endogenous muscle cathepsins, Seifter and Harper (1970) reported that denatured collagen is susceptible to the action of proteolytic enzymes.

Differences in tenderness attributable to heating rate may result partially from differing degrees of enzymatic breakdown of collagen. The greatest decrease in shear values occurred between 50 and 60°C. This decrease in shear values was paralleled by an increase in enzymatic activity. The time in this temperature range was greater for slowly heated cores. Therefore, an opportunity for prolonged activity in the slowly heated cores could result in differences in the breakdown of collagen. The curvilinear trend (Fig. 2) suggests that more than one proteolytic enzyme system might have been operative in the muscle samples. One system was inactivated during heating to approximately 44°C. A second system increased in activity to a maximum value at 65°C followed by a gradual decrease in activity. Lutalo-Bosa and MacRae (1969) observed proteolytic activity in beef muscle extract incubated above 60°C and suggested that an enzyme similar to cathepsin C which is heat stable at 65°C was responsible. Other cathepsins are inactivated above 60°C. The collagen shrinkage temperature or point of denaturation has been listed by several (Machlik and Draudt, 1963; Hamm, 1966) as 63°C. At that temperature the collagen would become susceptible to proteolytic activity of an enzyme such as the one described by Lutalo-Bosa and MacRae (1969). In the present investigation, time in the 60–70°C interval was 5.4 times (Table 1) as long for the slow rate as for the fast rate of heating allowing greater opportunity for enzymatic degradation. The possibility that enzymatic activity during the long period of heating from 60–70°C resulted in increased collagen solubilization in slow 70°C samples was not disproved in this investigation.

Solubilization of hydroxyproline in buffer

Little or no solubilization of hydroxyproline from CCT was evident in the 40 and 50°C buffer samples (Table 1). Evidently soluble hydroxyproline containing materials that were found in the intact cores heated to these temperatures were removed in the process of isolating the CCT from raw muscle tissue. Or, a non-heat related reaction was affecting solubilization of collagen from the beginning of the heating period to 50°C in the in-

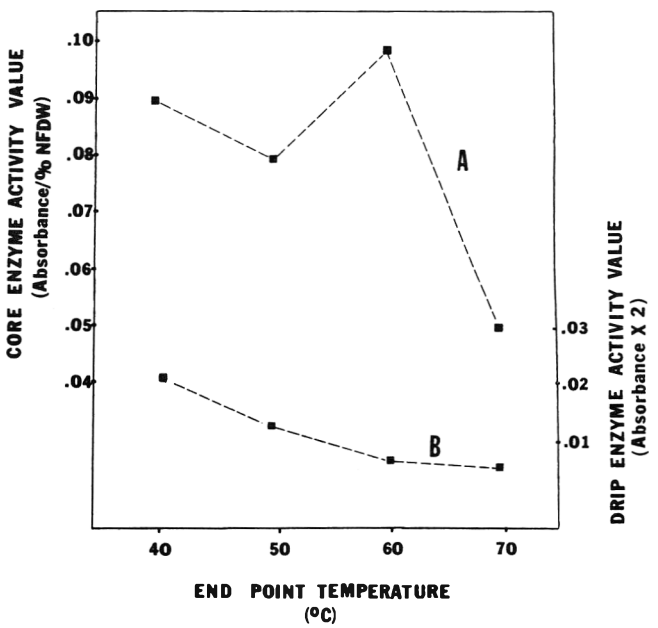

Fig. 3—The effect of end point temperature on proteolytic activity of cores (A) and drip (B).

tact meat system. Enzyme action might be a possibility.

Differences ($P < 0.001$) with respect to heating rate and end point were found (Table 2). Solubilization of 7.48 ± 1.40% hydroxyproline in the buffer system at the slow rate without respect to end point was greater ($P < 0.001$) than the 3.43 ± 0.53% solubilized in samples heated at the fast rate. Heating to 70°C at both rates solubilized 8.66 ± 1.10% of the hydroxyproline in CCT as compared to 2.24 ± 0.26% in the 60°C samples. The effects of heating rate and end point on solubilization of hydroxyproline were interrelated ($P < 0.001$).

Solubility in guanidine hydrochloride

Treatment means for yield of GSIC are presented in Table 1. Samples heated at the slower rate yielded less GSIC ($P < 0.05$) than the samples heated at the faster rate.

End point also had a significant effect on solubility of heated CCT in guanidine hydrocholoride. CCT heated to 70°C was less soluble ($P < 0.001$) than that heated to the lower end points (Table 2). The decreased yield of GSIC from 70°C samples might have resulted from solubilization of portions of it during heating or by conversion to a less soluble form. Solubilization of portions of GSIC would require breakage of cross linkages, both intra and inter molecular to form the more soluble α and β components of collagen or breakage of peptide linkages to form shorter sections of cross linked peptides.

From this study it appears that factors in addition to collagen solubilization are responsible for the increased tenderization of meat promoted by slower rates of heating. Endogenous proteolytic enzymes may play a role in tenderization during heating. Further characterization of the insoluble connective tissue residues after heating might help to explain the role of connective tissue and changes in it during tenderization of meat.

A comparison of the effects of different rates of heating on the myofibrillar components might also help to explain the increase in tenderness that occurs with slow rates of heating.

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FUNCTIONAL PROPERTIES OF PROTEINS ISOLATED FROM BOVINE BLOOD BY A CONTINUOUS PILOT PROCESS

INTRODUCTION

PROTEIN ISOLATES prepared from slaughter animal blood represent a potential source of large quantities of dietary protein. The proteinaceous material of whole blood was processed by Vickery (1968) to produce an isolate which was bland in taste, virtually odorless and contained 86% protein. Pals (1970) prepared an isolate from bovine plasma by spray drying the aqueous plasma after centrifugal separation. The isolate was light tan in color and contained 90% protein.

Tybor et al. (1973) described a process for the preparation of a plasma and globin isolate from slaughter blood. The red cells were concentrated by centrifugal separation of whole blood, and hemolyzed hypotonically. The hemoglobin was converted to choleglobin from which the porphyrin could easily be removed in an acidified acetone solution. The plasma and globin protein fractions were desalted and spray dried to produce white, free-flowing isolates.

Protein concentrates prepared from whole blood are excellent emulsifiers (Satterlee et al., 1973). The whole blood proteins exhibit emulsification capacities and emulsion stabilities equal to or greater than that of the proteins of other organ and tissue concentrates including muscle proteins. Plasma and globin protein isolates prepared from slaughter blood are excellent emulsifiers under optimum conditions of pH and protein concentration (Tybor et al., 1973). The emulsification capacity of the plasma isolate is particularly sensitive to processing conditions. The proteins in the globin isolate were good emulsifiers when compared to nonfat dry milk and the single sample of cottonseed isolate studied by Crenwelge et al. (1974).

The purpose of this investigation was to evaluate the functional properties of plasma and globin protein isolates prepared by a continuous process which would be adaptable to commercial operations.

EXPERIMENTAL

Preparation of plasma and globin protein isolates

Whole bovine blood was collected at the time of slaughter and commingled (9:1/v:v) with 0.85% NaCl solution containing sufficient sodium citrate to provide a level of 0.02% in the mixture. The mixture was maintained at 5°C and separated within 24 hr into the plasma and red blood cell fractions with a portable milk separator. The process for the preparation of isolates from the whole blood is presented in Figure 1. The aqueous plasma was spray dried after this operation without further processing.

Globin was isolated from hemoglobin by a continuous pilot-scale process. The schematic diagram for this process is presented in Figure 2. Ascorbic acid was added to the hemoglobin solution in a 55-gal stainless steel vat to bring the solution to pH 4.0. The hemoglobin-ascorbic acid suspension was pumped through 1.5 in. sanitary pipe line into a high speed turbomixer which was mounted on a 4 in. s.s. tee equipped with a baffle plate on the outlet of the tee. This baffle plate allowed for greater dwell time in the mixer where the hemoglobin was converted to choleglobin. The mixer was operated at 2500 rpm. The chromoprotein solution was then passed into a second mixer assembly where acidified acetone was introduced into the protein solution at a 4:1 (v:v) ratio. This assembly was equipped with a baffle plate at the inlet and outlet and the mixer was operated at 5000

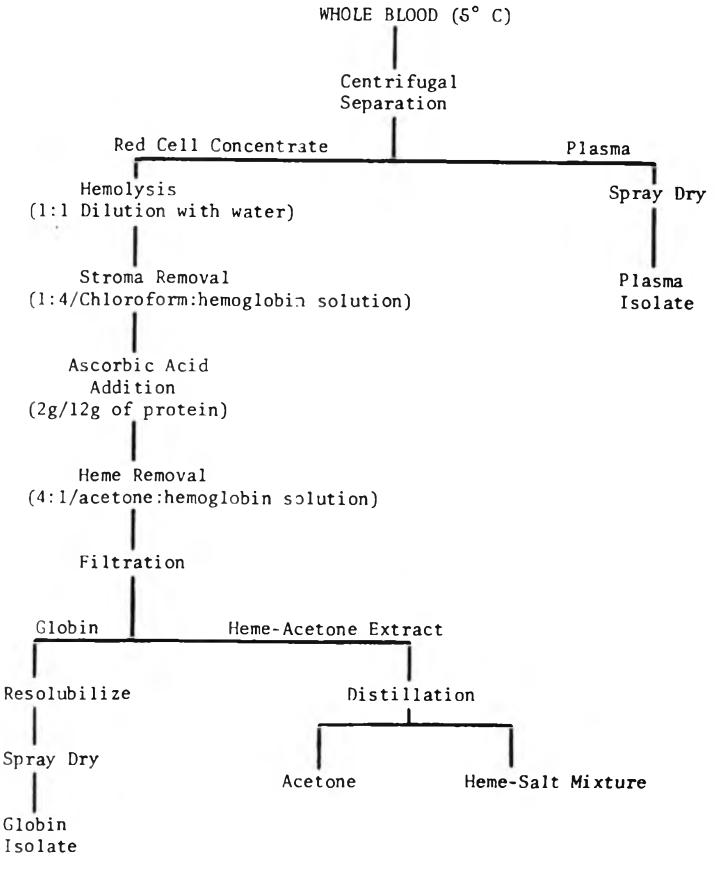

Fig. 1—Flow diagram of the process for preparing blood protein isolates.

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Table 1—Composition of spray-dried plasma and globin isolates

Sample	Moisture	Protein	Na	Cl	K	Ca	Mg
				%			
Plasma	2.47	70.88	8.50	9.93	0.35	0.10	0.03
Globin	3.41	91.22	0.60	5.10	0.03	0.03	0.02

Table 2—Amino acid composition of spray-dried plasma and globin proteins

Amino acids	Amino acid composition		
	Globin	Plasma	1957 FAO provisional pattern of essential amino acids[a]
	g/100g of protein		
Essential			
Lysine	10.5	9.2	4.2
Threonine	3.8	6.3	2.8
Methionine	1.7	1.0	2.2
Valine	9.4	7.0	4.2
Phenylalanine	7.9	5.6	2.8
Leucine	13.8	10.1	4.8
Isoleucine	0.2	2.9	4.2
Tryptophan	2.0[b]	1.9[b]	1.4
Histidine	7.8	3.5	–
Nonessential			
Arginine	3.6	5.0	
Aspartic acid	10.0	10.7	
Serine	3.0	5.5	
Glutamic acid	6.8	13.7	
Proline	3.5	3.8	
Glycine	3.7	3.6	
Alanine	8.6	5.3	
Cysteine	0.1	1.2	
Tyrosine	2.5	3.6	

[a] FAO/WHO (1965). Levels suggested for human adults.
[b] These data were provided by C.R. Young, Dept. of Biochemistry and Biophysics, Texas A&M University, College Station.

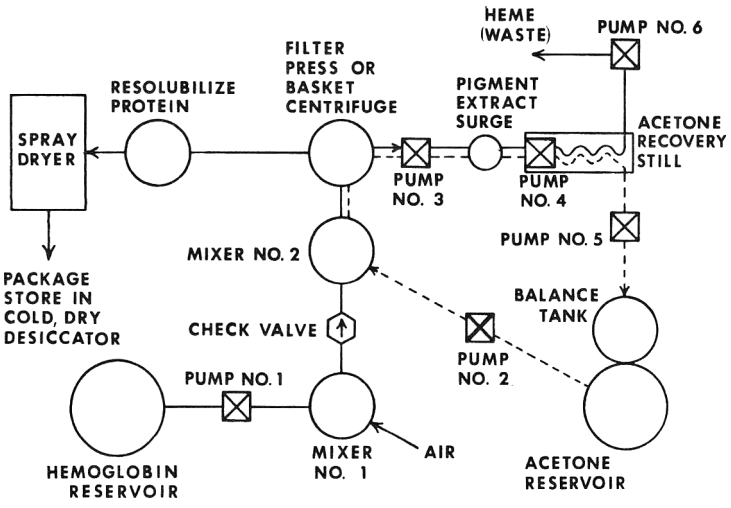

Fig. 2—Schematic layout of the continuous system for decolorizing hemoglobin.

rpm. The prosthetic group of the chromoproteins was removed and the globin proteins precipitated during this phase of the process. The protein slurry was collected, by filtration through unbleached muslin, washed with additional acidified acetone, resolubilized in water and spray dried to yield a white and free-flowing globin protein isolate.

The powdered isolates were individually packaged and stored under vacuum with desiccant. The heme-acetone extract was distilled using a Precision laboratory evaporator to regenerate the acetone.

Proximate analysis

The gross composition of plasma and globin protein isolates were determined by methods described by AOAC (1970). Protein, moisture, sodium, chloride, magnesium, calcium and potassium were included in this analysis. Kjeldahl nitrogen was converted to percent protein using the factor 6.35. Chloride and calcium were estimated by titration while sodium, potassium and magnesium were determined by atomic absorption spectrophotometry.

Amino acid and microbiological analysis

The amino acids were separated and analyzed on a Beckman 121 C Amino Acid Analyzer, by the basic procedure of Moore and Stein, (1954).

The microbial quality of plasma and globin protein isolates was analyzed by the methods prescribed by USDHEW (1969). Total aerobic organisms and pathogens (salmonella, shigella, and staphylococci) were determined.

Solubility

The solubility of the plasma and globin isolate proteins was determined by the method of Lawhon and Cater (1971) with modifications. One percent stock solutions of the proteins were prepared with distilled-deionized water and aliquots adjusted to pH values ranging from pH 2.5–9.5 with 1N HCl or 1N NaOH. The pH of the globin solutions were allowed to equilibrate overnight at 5°C and readjusted if necessary. Total protein and water soluble protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Emulsification capacity

The emulsification capacity of the protein isolates was determined according to the method of Tybor et al. (1973). An aqueous phase volume of 100 ml and an initial rate of oil addition of 40 ml/min were used for the analysis.

Foaming properties

The foaming capacity and foam stability of isolate proteins were evaluated by the method of Lawhon and Cater (1971). The isolates were solubilized in 50 ml of distilled-deionized water and their foaming capacity determined as a function of pH and protein concentration.

Foaming stability of the plasma and globin protein isolates was determined over a 10-min time interval and compared to that of egg albumen. The egg albumen was prepared fresh from the eggs of White Leghorn chickens which were 240 days old and on an 18% protein ration.

RESULTS & DISCUSSION

Composition of plasma and globin protein isolates

The major organic and inorganic nutri-

Table 3–Plate counts for the total aerobic and pathogenic organisms in spray-dried plasma and globin isolates

Analysis	Plate counts of protein isolates	
	Plasma	Globin
	organisms/g	
Total aerobic counts	<300	<300
Salmonella	< 10	< 10
Shigella	< 10	< 10
Staphylococci	< 10	< 10

ents in the plasma and globin protein isolates are presented in Table 1. The proteinaceous material accounted for approximately 71 and 91% of the plasma and globin isolate weight, respectively. Less than 0.1% lipid could be extracted (AOAC, 1970) from the plasma isolate and none was detectable in the globin isolate. The plasma contained high levels of sodium and chloride relative to that of the globin. The chloride concentration of the globin is disproportionately larger than the level of sodium. Since sodium citrate was added at the time of collection to the whole blood, it is a constituent of the plasma. The sodium in excess of that accounted for as NaCl was assumed to originate with the citrate. Calculations based on this assumption indicated that citrate accounted for 8.61% of the isolate weight. The inclusion of this value with those in Table 1 brings the sum of the plasma components to 100%.

Amino acid analysis of the spray-dried plasma and globin isolates (Table 2) indicates that both isolates contain all of the amino acids essential to human nutrition. The isolates are excellent sources of lysine and leucine. The levels of other essential amino acids (threonine, valine, phenylalanine and tryptophan) are greater than the levels proposed by FAO/WHO (1965) and are indicative of good nutritive value. However, isoleucine and methionine are limiting amino acids for both protein isolates. Although levels of isoleucine in the globin is of particular concern (Young et al., 1973), the globin is characterized by a relatively high level of histidine, which is required by human infants (Clark, 1965).

Microbiological quality

Plasma and globin protein isolates which were spray dried at 160°C were analyzed for their bacterial content. The numbers of total aerobic organisms, salmonella, shigella, and staphylococci contained in the protein isolates are presented in Table 3. Each isolate contained less than 300 viable aerobic organisms per gram. Bacterial colonies indicating the presence of salmonella, shigella or staphylococci were not observed on any plate inoculated with the 1:10 dilution of the protein isolates. Since blood in its natural state is usually sterile (Richards, 1970), the excellent microbial quality of these plasma and globin isolates was not unexpected. The incidence of aerobic bacteria probably was due to contamination during collection, processing and post-processing procedures.

Solubility of the plasma and globin protein isolates

Plasma and globin protein isolates were spray dried at 160° or 193°C with and without lactose to determine the effect on the solubility of the protein isolates of drying temperature and lactose incorporation prior to drying. The lactose was added to the aqueous protein suspensions (1:1/protein:lactose) prior to spray drying.

The effect of drying temperature and lactose incorporation on plasma protein solubility is presented in Figure 3. The plasma control exhibited greater than 90% solubility at pH values ⩽ 4.0 ⩾ pH 6.0. The point of minimum solubility occurred at pH 4.8 where approximately 74% of the protein was soluble. Tybor et al. (1973) demonstrated that the solubility of the plasma proteins essentially was little affected by changes in pH while the globin protein was most soluble below pH 6.0. The plasma proteins dried at 160° and 193°C exhibited a 20% reduction in soluble protein relative to the control which was a sample of raw, lyophilized plasma. Jenness and Patton (1959) indicated that the temperature of the particles during spray drying never exceeds

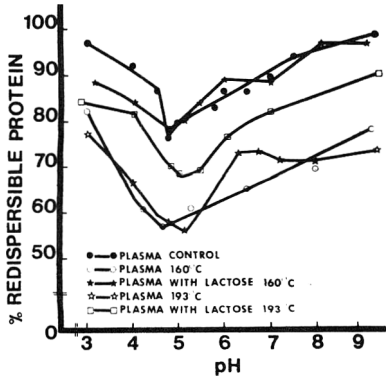

Fig. 3–Effect of drying temperature and lactose incorporation on the solubility of the plasma proteins.

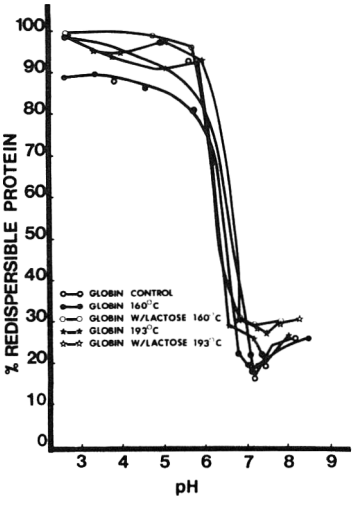

Fig. 4–Effect of drying temperature and lactose incorporation on the solubility of globin concentrates prepared by the continuous heme removal process.

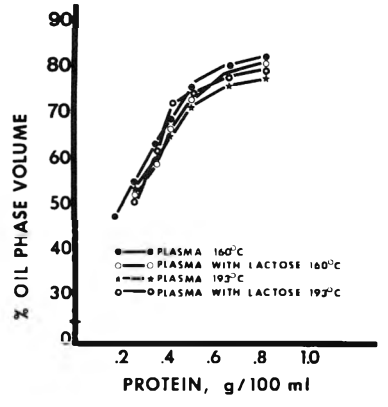

Fig. 5–Effect of drying temperature and lactose incorporation on the emulsification capacity of the plasma proteins.

Table 4—Foam volumes for egg albumen, plasma and globin isolates at 0, 2, 5 and 10 min

Sample	Foam volume (ml) V_0^a	V_2	V_5	V_{10}	$\%\Delta V^b$
Globin pH 2.5	185	174	169	165	12.0
Globin pH 6.0	198	188	183	182	8.0
Globin pH 7.2	146	135	120	118	19.0
Plasma pH 9.6	108	90	84	80	26.0
Plasma pH 4.8	131	116	111	108	17.5
Egg albumen (undiluted)	108	106	104	101	6.5
Egg albumen (diluted)	121	106	101	100	17.0

[a] Subscripts indicate the time of foam volume measurement.
[b] Foam stability = $\%\Delta V = [(V_0 - V_t)100]/V_0$.

the air outlet temperature. Since this temperature was the same for both isolates (107°C), the decreased solubility probably is representative of the effect of air outlet temperature and explains why solubility differences between the two drying temperatures were not observed. Regardless of the temperature effect, the incorporation of lactose prior to drying demonstrated a protective effect on protein solubility. This corroborates results noted by Sugimoto and Van Buren (1971) in their work with soy proteins. Solubility was greater at both drying temperatures when lactose was incorporated. However, the greatest effect occurred when the proteins were dried at 160°C where their solubility was essentially the same as the lyophilized plasma control. Lactose is believed to prevent disulfide polymerization of the proteins and thereby enhance solubility (Sugimoto and Van Buren, 1971). However, the proteins dried at 193°C exhibited a point of minimum solubility at pH 5.1, which is 0.3 units greater than the other isolates, and indicates that lactose was not effective in preventing this change.

Figure 4 summarizes the effect of drying temperature and lactose incorporation on the solubility of the globin isolate proteins. All of the globin protein isolates displayed maximum solubility at values ≤ pH 6.0. With the exception of the globin dried at 160°C, the isolates exhibited from 90–100% solubility over this pH range. Minimum protein solubility was observed at pH values ranging from pH 7.0–7.5. Neither drying temperature nor lactose incorporation had a significant effect on globin protein solubility. However, the point of minimum solubility was different (pH 7.4 as compared to pH 7.2) when the isolate was dried at 193°C.

Emulsification capacity of the protein isolates

The emulsification capacity of the plasma and globin protein isolates was investigated to determine the effect of drying temperature and lactose incorporation on this property. Results for plasma proteins are presented in Figure 5 and the globin proteins in Figure 6. The emulsions were formed at pH 9.4 where the emulsification capacity of processed sera exhibited the best response to protein concentration (Tybor et al., 1973). The response of the plasma proteins to protein concentration was hyperbolic as previously noted (Tybor et al., 1973) for the lyophilized control serum. All protein isolates approached the theoretical oil phase maximum (Becher, 1965) at a protein concentration of 0.5g/100 ml. However, neither a temperature nor a lactose effect on emulsification capacity was observed.

The globin proteins exhibited a sigmoidal response to protein concentration which was similar to that described by Tybor et al. (1973) and Crenwelge et al. (1974). The theoretical oil phase maximum (74%) is surpassed at a protein concentration of 0.4g/100 ml. The emulsification capacity of the globin proteins also showed no effect for drying temperature and lactose incorporation.

Foaming properties

The effect of protein concentration on the foaming capacity of the blood protein isolates and egg albumen is presented in Figure 7. The response of all three proteins is hyperbolic. The maximum foaming capacity for the plasma and globin

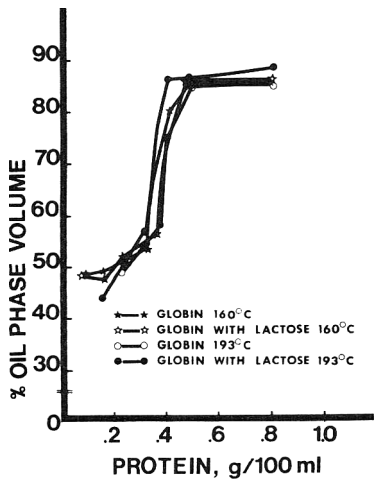

Fig. 6—Effect of drying temperature and lactose incorporation on the emulsification capacity of globin proteins.

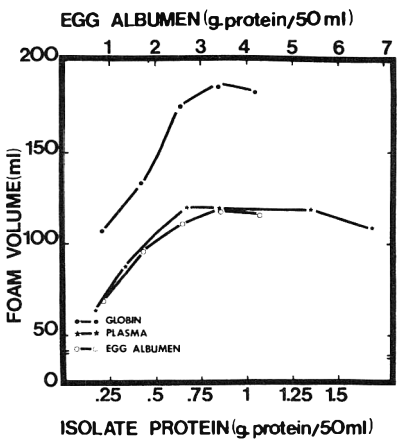

Fig. 7—Effect of protein concentration on the foaming capacities of egg albumin plasma proteins and globin isolate proteins.

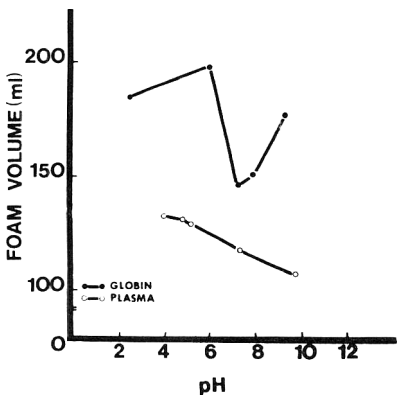

Fig. 8—Effect of pH on the foaming capacity of plasma and globin isolate proteins.

protein isolates was observed at a protein concentration of 1.70g/100 ml. The maximum foam volumes for the plasma and globin were 120 ml and 187 ml, respectively. The foaming capacity of the plasma was equivalent to that of the albumen at each protein concentration.

The effect of pH on the foaming capacity of the plasma and globin protein isolates is presented in Figure 8. These foams were formed using 1.7g of protein/100 ml which was shown to be the optimum protein level (Fig. 7). The plasma isolate proteins exhibited a linear response to pH. The foam volume decreased from 133 ml at pH 4.0 to 108 ml at pH 9.6. The foaming capacity of the globin proteins was greater than that of the plasma over all pH values studied. The response of the globin isolate to changes in pH was similar to its solubility profile (Fig. 4). The globin isolate proteins exhibited a maximum foaming capacity at pH 6.0 (197 ml) and a minimum at pH 7.2 (145 ml).

Table 4 summarizes the foam stability of the albumen and the plasma and globin isolates. The plasma and globin foams were prepared at 1.7g of protein/100 ml. Albumen foams were prepared using undiluted albumen which contained 13.5g protein/100 ml and a diluted albumen (5.4g/100 ml). The stability of the globin foams were studied at pH 2.5, 6.0 and 7.2 while the plasma pH values were 4.8 and 9.6. These pH values correspond to points of high and low foaming capacities observed in Figure 8. The foam volumes for all protein samples decreased over the 10-min quiescent period. The globin (pH 6.0) exhibited the most stable foam volume over this time period. Among samples of the same protein source, those with the greatest initial foam volume exhibited the least degree of volume change. However, the dilute albumen, which had the greatest foam volume of the two albumen concentrations, was least stable. A comparison of percent change in foam volume indicated the adequacy of the protein isolates as foaming agents. The undiluted albumen exhibited a 6.5% change over the 10 min period. The change observed for the plasma proteins and the globin (pH 7.2) was three to four times greater than that of the natural albumen. The stability of the globin (pH 2.5) was approximately half that of the undiluted albumen while the stability of the globin foam prepared at pH 6.0 was comparable to the natural albumen.

CONCLUSIONS

PLASMA and globin protein isolates are a source of large quantities of protein which has potential as a nutrient in human foods. Isoleucine and methionine are limiting amino acids. These isolates are microbiologically safe as indicated by the low numbers of aerobic bacteria and the absence of pathogenic microorganisms.

Functionally, the plasma isolate proteins are more responsive to drying treatments than the globin proteins. The solubility of the plasma proteins is reduced during the spray drying operation. However, lactose can be incorporated prior to drying to maintain the solubility characteristics of the proteins. Both the plasma and globin protein isolates are excellent emulsifiers based on data for model systems presented here, and are independent of drying conditions with respect to this function. These protein isolates are also good foaming agents under optimum conditions of protein concentration and pH. The globin exhibits a greater foaming capacity and better stability than the plasma and seems to possess greater potential for use in foods as a foaming agent.

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PROTEIN-LIPID FILMS AS MEAT SUBSTITUTES

INTRODUCTION

DURING THE 1960's an intensive worldwide effort in institutional and industrial laboratories resulted in the emergence of food or potential food proteins from nontraditional or under-utilized raw materials (Polopolus, 1970; Wilding, 1971). Concurrent with these developments have been efforts to present these proteins in acceptable textural form resembling traditional animal protein foodstuffs.

The commercial soundness of such an approach is demonstrated by the viability of a range of "first generation" textured protein foods which derived their tactile properties from ingenious production and manipulation techniques. Successful processes include protein fiber spinning (Giddey, 1965; Ziemba, 1969; 1971), extrusion (Sanderude and Ziemba, 1968; Martin et al., 1971), protein curd manipulations (Pinkston and Claydon, 1971), and pressure forming of natural protein scraps (Fenters and Ziemba, 1971; Learson et al., 1971). Hydrocolloid interaction also shows promise of providing desired texture to a wide range of food systems, including protein foods (Andrews and MacLeod, 1970).

The commerical base of textured protein foods is primarily soybean due to the favorable economics and excellent protein functionality of this material (Smith and Wolf, 1961; Corey, 1970). However, analogous utilization research with a range of protein sources is continuing at an accelerated pace, and it is probable that other proteins and processes will complement and compete with soybean within the next decade (Gibson and Dwivedi, 1970; Heden et al., 1971).

One attractive process for producing textured protein foods not presently receiving the attention it merits is yuba formation, in spite of the fact that yuba has been the basis for traditional Oriental foods for centuries (Borgstrom, 1969). Yuba is the surface protein-lipid film which forms on soymilk upon heating. Recent investigations on yuba formation, employing soymilk and model protein-lipid systems have given some insight into film formation mechanics and conditions for optimizing film yield, and has been extended to other protein sources such as peanut, cottonseed, whey and milk (Wu and Bates, 1972a, b; 1973).

Traditional methods of film formation as performed in the Orient consist of heating soymilk in shallow pans and periodically removing the films manually when their strength so warrants. Films are hung to air dry as sheets or rolls. Fabrication is performed with rehydrated and flavored films. The sheets are soaked in appropriate flavoring solutions such as soy or meat broths, layered several sheets thick, rolled tightly, wrapped firmly in cloth, and tied to retain internal pressure. The rolls are then steamed for about 1 hr and consumed as a main dish. An alternate texturization process involves placing layers of moist, flavored films in aluminum molds shaped like whole chicken or fish. The center of the mold may be stuffed with film remnants, or fitted with a wooden plug, thus providing a hollow space for subsequent stuffing ingredients. The mold is closed and manual pressure applied, resulting in a firm meat-like texture of desired shape.

The high level of acceptability of these yuba products is due to a combination of pleasing texture and flavor characteristics. Although they employ low-cost raw materials, extensive hand labor is required. Consequently, such fabrication techniques are not conducive to the production of uniform quality, high volume food materials. This report is based upon subsequent studies of protein-lipid film formation employing film production and fabrication techniques as steps in the development of useful textured vegetable protein foods.

EXPERIMENTAL

RAW MATERIALS employed as protein-lipid film ingredients, oilseed extraction, preparation of dispersions, adjustments of composition and concentrations, film yield evaluations and analyses were as previously reported (Wu and Bates, 1973).

The semi-continuous film formation and recovery system, shown schematically in Figure 1, consisted of a flat shallow stainless steel pan (1.20 × 0.33 × 0.08 m) with inlet and outlet ports at the ends. The inlet stream was distributed over the width of the pan through a T-shaped spreader. The outlet was connected to a positive displacement pump feeding a 1 ft^2 Votator Scraped Surface Heat Exchanger operated on 10 psi steam. This in turn was connected to the inlet end of the pan. Flow rate was maintained at about 4 liters/min and fluid temperature at 95 ± 5°C. A small piece of plastic screen running the width and depth of the pan was fixed in front of the inlet T spreader to prevent foam from floating onto the 0.36m^2 film formation surface. An electric fan blowing at slow speed (10 ft/sec) was set 2m behind the intlet end to air surface dehydration and thus speed up film formation. The film take-up reel consisted of a 1/2 in. o.d. stainless steel rod suspended parallel to and 5–15 cm above the film surface. Film recovery consisted of manually rolling it up on the take-up reel or, for partial drying, by slipping a rod under the film surface at the center of the pan and gently lifting the film sheet from the surface. The sheet was then air dried at 50°C to various moisture contents.

When desired, various flavors were imparted to the films by sprinkling powdered or liquid flavorings and spices onto the film surface prior to withdrawal. Film fabrication and manipulation experiments were performed mostly on

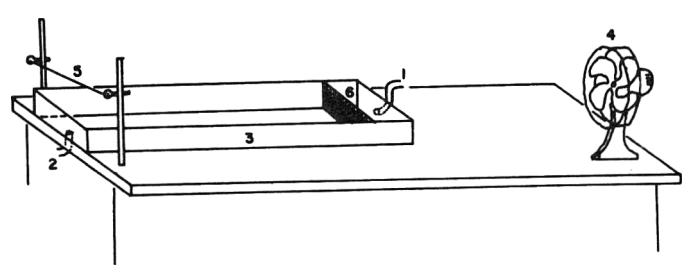

Fig. 1—Schematic diagram of the prototype film formation device. [(1) Inlet from votator; (2) outlet to pump; (3) stainless steel pan (1.20 X 0.33 X 0.08 m); (4) fan; (5) take-up reel; (6) plastic screen.]

Table 1—Protein incorporation efficiencies[a] of films from various raw materials under continuous or batch conditions.

Raw material	Film protein (% db)	Protein incorporation efficiency (PIE)		
		Continuous	Batch	Traditional
SPI	83.4	65.2	68.1	–
SPI & oil (2/1)	58.5	76.0	71.4	–
Soybean	52.4	82.3	84.0	65.0
Peanut	31.4	70.0	–	–
Whole milk	31.0	71.2	–	–
NFDM	57.4	79.3	–	–
Glandless cottonseed	40.0	37.5	–	–

[a] (Wt protein in dispersion/wt protein in film) X 100

fresh soy film rolls. Approximately 200g cylindrical sections were cut longitudinally from the rolls, flattened, formed into rectangular slabs about 9 × 6 × 4 cm and tightly wrapped with transparent plastic (Handi-Wrap) wrapping film. The film slabs were then placed in individual 20 × 15 cm polyester/aluminum/nylon laminate pouches (Goldfarb, 1970) and sealed under vacuum.

Thermal treatments consisted of retorting the pouches at various temperature-time combinations. Overriding air pressure was required only during water cooling to prevent pouch rupture.

In addition to the rolled, flattened films, randomly oriented (torn and crumpled) film pieces or layers of partially dried films were formed into slabs of similar geometry in order to evaluate other ways of film alignment. Film moisture content was adjusted by air drying or soaking films in water at 2°C until they imbibed sufficient moisture.

Fabricated film slabs were evaluated in duplicate by the authors for appearance by reviewing a cross section of the slab (Figure 2) and for texture by handling and then chewing a portion of the slab and noting the feel and mouthfeel characteristics according to criteria described in Table 1.

RESULTS & DISCUSSION

THE SEMI-CONTINUOUS system of film formation was a marked improvement over prior batch formation methods. Protein incorporation efficiencies (PIE, protein in films/total protein in dispersion) were similar to those obtained in batch experiments and superior to the traditional process (Table 1). Advantages included labor-saving, less coagulation of solids on the walls and bottom of the pan, ease of adding make-up components—water or protein-lipid dispersion—and increased film formation rate due to larger surface area.

While considerable design improvements can still be effected by attention to film formation kinetics, heat transfer and conservation, fluid and air flow considerations and film withdrawal and handling techniques, this prototype system resulted in efficient fabrication of abundant film material for subsequent experiments and a film dry weight formation rate of about 6g/min-m^2.

Preliminary experience with yuba formation employing traditional fabrication methods—wrapping layered films tightly in cloth, binding with string and steaming—indicated that formation of a coherent mass was dependent on both heat and tight binding. Otherwise, the films retained their identity and separated readily when handled. Whereas, with the application of both heat and pressure, the films fused and formed a continuous, firm textured mass which held together well when sliced or handled.

The foil pouches served to maintain the structure and shape of the film during manipulation and provided a sanitary package. A number of slab sizes and shapes were considered and the 9 X 6 X 4 cm, 200g size was chosen as an appropriate amount to handle and evaluate.

Although the evaluation criteria (Table 2) were arbitrary and subjective, tests were quite simple to perform and standardize. Freshly collected soy film rolls had an appearance score of 0 (Fig. 2) and a texture score of 1. Acceptable texture values ranged between 3.5–5.0. Below 3.5, film slabs were too soft to function as a discrete food entity, behaving much like over-cooked pasta. Above 5.0, slabs were overly tough and chewy.

Increasing process time or temperature had a beneficial effect upon slab appearance. Although heat penetration was quite slow (slabs required 60 min to reach a center temperature of 85°C when steamed at 100°C), there was no evidence of this time-temperature differential in a cross section of the slab. The surface layers did not appear different from the interior, except in milk films where a pronounced browning of the surface was noted.

In contrast to the appearance, the texture score was much more time-temperature dependent (Fig. 3). Prolonged heating at either 130 or 121°C resulted in serious texture breakdown in the slabs. In addition, a slight scorched flavor was noted in slabs heated for more than 15 min at 130°C or 25 min at 121°C. Thus, 121°C for 20 min was adopted as the standard process and used most widely. However, this process corresponded to a slab center F_0 of less than 1 min due to slow heat penetration, so the film slabs were, at best, lightly pasteurized. For

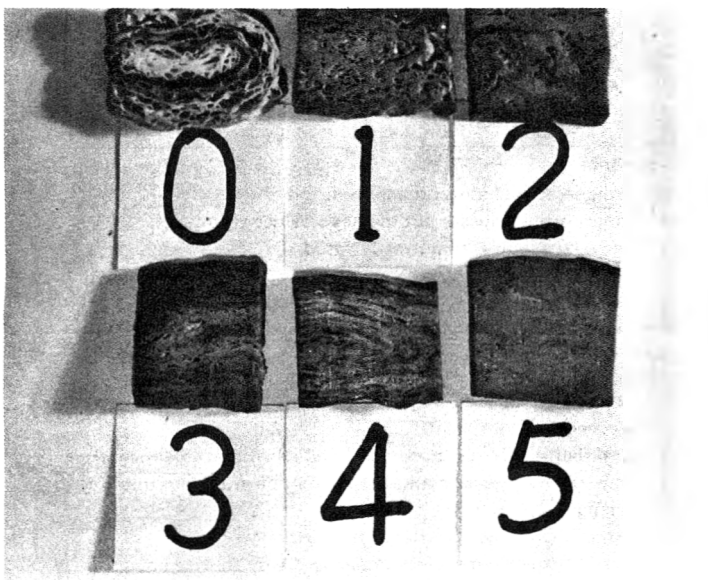

Fig. 2—The cross sectional appearance scores assigned to fabricated soybean film slabs.

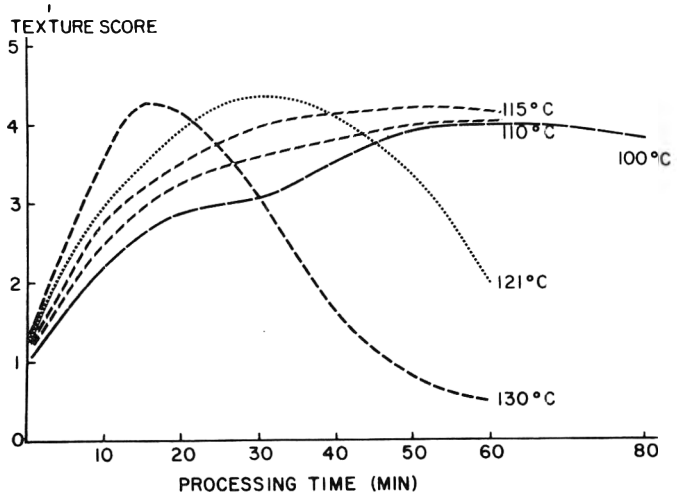

Fig. 3—Texture score of fabricated soybean film slabs as affected by temperature and processing time.

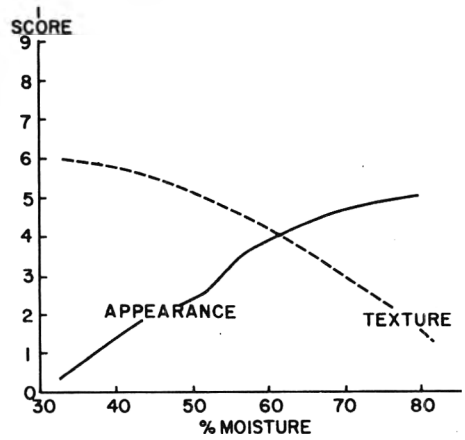

Fig. 4—Influence of moisture content on appearance and texture of soybean film slabs (121°C – 20 min).

large-scale production, continuous film slab formation and processing would be the logical approach, but the pouch technique provided useful insights into the fabrication phenomenon.

In an attempt to reduce the time or temperature required for optimum texture development, trials were conducted in which a vented pouch was subjected to up to 5 psi mechanical pressure under various processing conditions. However, 5 psi had only a slight positive effect upon appearance and none upon texture. Also, although higher mechanical pressures might be beneficial, ingredient extrusion from the pouch made further testing impractical. The effect of film alignment was pronounced. At all processing conditions tested, the appearance and texture of products from randomly oriented film pieces were poorest, that of film rolls intermediate and the orderly layered partially-dried-film slabs best. Partially dried films had the best texture. Loose film scraps and films collected by simply scraping from the formation surface can be used for slab fabrication, although discrete films perform better.

The most influential variable affecting both slab texture and appearance was film moisture content (Fig. 4). Freshly formed, drained soy films had a moisture content of about 66%. It was necessary to condition (dry or hydrate) single film sheets prior to slab formation, since after rolling or layering, the films were very good barriers to moisture diffusion.

Moist film slabs, being more pliable, formed a more continuous structure with few voids and a good appearance. These slabs, however, had low texture ratings and disintegrated upon handling. On the other hand, drier films did not fuse together as well, but the slabs produced a much firmer texture; in some cases actually rubbery. In general, for soy films, a moisture content of 50–70% represents a good range for fabrication, depending upon the desired texture of the end product.

All slabs were evaluated at about 25°C, but in order to establish the effect of temperature, some slabs were evaluated as they cooled down from the process temperature of 121°C. Appearance was relatively unaffected, but a marked reduction in texture was noted above 60°C. Since this breakdown in slab integrity occurred above a tolerable eating temperature, it is a limitation only during cooking and serving. Thus, vigorous agitation during preparation should be minimized.

The influence of other protein or lipid sources is shown in Table 3. Increasing film lipid or decreasing protein contents adversely affect slab texture. Coherent slabs were unattainable from peanut or peanut:soybean (3:1) films. However, partial dehydration of films prior to slab formation can compensate somewhat for the low protein:lipid ratio, provided that the films are strong enough to be recovered from the dispersion surface. The slabs made from soy films withdrawn toward the end of a run were somewhat tougher than those withdrawn earlier, due probably to an increase in the protein:lipid ratio. However, the intrinsic prop-

Table 2—Film slab evaluation criteria

Appearance

0 = films separated as in original film roll
1 = films partially fused, but many voids between layers
2 = a few large voids, film mostly fused
3 = many small voids
4 = a few small voids, film almost fused together
5 = individual films cannot be distinguished, several small voids
6 = a totally solidified cross section

Texture

0 = a puree-like consistency
1 = film slab is soft, can barely be handled without disintegrating
2 = consistency of over-cooked pasta, but disintegrates upon slicing
3 = firmer than 2, can be sliced and handled with care
4 = elastic firmness, meat-like chewiness, easy to slice and manipulate
5 = slightly rubbery, but not tough
6 = rubbery, tough, chews like tough meat

Table 3—Appearance (A) and texture (T) scores of various finished products fabricated at 121°C for 20 min

		Wet fresh roll			Partiality dried and rearranged		
	P/L[a]	Moisture	Score		Moisture	Score	
Raw material	Ratio	(%)	A	T	(%)	A	T
Soybean I[b]	2.3	64.5	4.0	3.7	56.5	4.0	4.0
Soybean IV[c]	2.9	55.9	4.5	4.5	–	–	–
SPI	103.8	74.0	3.0	3.5	66.6	5.0	5.0
SPI & oil (3:1)	3.7	68.7	2.5	3.5	62.0	3.0	4.0
SPI & oil (2:1)	2.3	67.6	2.5	3.0	57.8	3.5	4.0
SPI & oil (1:1)	1.5	62.5	2.5	2.0	52.3	3.0	4.0
Peanut	0.6	46.2	2.5	2.0	–	–	–
Peanut & soybean (3:1)	0.7	49.2	3.0	2.5	–	–	–
Peanut & soybean (1:1)	1.1	58.3	3.5	3.0	46.3	4.0	3.5
Nonfat dry milk	32.0	58.7	3.0	5.0	50.0	4.5	5.5
Whole milk	1.1	45.0	2.5	3.5	–	–	–
Glandless cottonseed	0.8	56.8	2.5	3.5	41.7	3.5	3.5

[a] Protein to lipid ratio
[b] First roll (light in color)
[c] Fourth roll (dark in color)

erties of the specific protein is also critical (Giddey, 1965) since milk films produced tougher slabs than oilseed films.

In order to gain some insight into the potential of protein:lipid films as meat substitutes, a number of film slabs possessing texture scores over 3.5 were prepared in various forms. Bouillon flavored slabs cut longitudinally into 2 cm thick sections and browned slightly in vegetable oil had the appearance and texture of the entree portion of a meat dish. The slab flavor was only vaguely reminiscent of meat, although not unpleasant. Slices about 3 mm thick functioned well as a meat loaf and chunks or dices also substituted well for meat ingredients in salads, casseroles and meat sauces. In these cases, flavoring with spices in a thick sauce or gravy was useful in flavor improvement.

Informal evaluation by lab personnel indicated that lack of "meat-like" flavor was the primary defect in film slabs prepared under optimum conditions from flavored soy, soy protein isolate (SPI) and milk films. The texture was deemed acceptable except for an easily correctable rubberiness in the SPI product. Since no obvious off-flavors were present, flavor enhancement can be effected once satisfactory texture is achieved.

While the "meat-like" nature of protein-lipid film products has been stressed, uses might eventually evolve in which such items could stand on their own merits without relating to meat foods. Such has been the case in the Orient with a range of soy derived textured foods. Pasta products are the only clear example in the United States.

The number of possible combinations of ingredients, process parameters and end product fabrications from protein-lipid films is enormous, and much basic and applied research will be required to systematically develop the state of the art of film utilization. However, the potential for this type of product is intriguing, and much of the evolving methodology in the flavoring, fabrication and utilization of meat analogs produced by extrusion, gelation, fiber formation, etc. is relevant to protein-lipid films.

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KINETIC ANALYSIS OF LIGHT-INDUCED RIBOFLAVIN LOSS IN WHOLE MILK

INTRODUCTION

OPTIMUM STORAGE conditions are important in maintaining the nutritional quality of liquid foods. These conditions include storage temperature, type of container and exposure of the product to various light intensities. The objective of this paper is to demonstrate the use of kinetic analysis in selecting the optimum storage conditions for maintaining maximum retention of a light-sensitive nutrient in a liquid food. With this overall objective in mind, riboflavin loss was monitored in whole milk stored under various conditions.

The deleterious effects of light on milk were first reviewed by Stull (1953). Aurand et al. (1966) reported the relationship between riboflavin and ascorbic acid on the development of "oxidized" flavor. Wishner (1964) reviewed the various photochemical alterations of nutrients that result in nutritionally poor quality of milk. Various investigators have reported the effects of fluorescent light on the flavor and nutrients in milk held in different types of containers (Dimick, 1973; Sattar and deMan, 1973; Hansen et al., 1972; Friedrich and Waiblinger, 1969; Radema, 1962). The mechanism of riboflavin photolysis was first proposed by Karrer et al. (1934). Under acid or neutral conditions, the product of the reaction is lumichrome, whereas in alkaline conditions, the product is lumiflavin. Both lumichrome and lumiflavin have no biological activity. The photolysis is irreversible and temperature-dependent.

The specific objectives of this study were (1) to evaluate kinetic parameters of riboflavin degradation in milk stored in various types of containers under different light and temperature conditions; and (2) use of these kinetic parameters in selecting the container that provides maximum retention to the vitamin.

EXPERIMENTAL

THE FOLLOWING TYPES of gallon-size containers were used in the investigation: (1) regular blow-molded polyethylene (BMP); (2) gold-pigmented blow-molded polyethylene; (3) paperboard; and (4) glass.

All storage experiments were conducted in controlled-temperature rooms. These rooms had a floor area of 1.5m by 1.5m and a ceiling height of approximately 2m. Each room had an evaporator coil to provide temperature control. Three storage temperatures were investigated, 1.7, 4.4 and 10°C. The temperature variation in the room was ±1°C.

Fluorescent tubes (General Electric, 90 watts, coolwhite) were used as the light source. A schematic layout of the containers being exposed to light is shown in Figure 1. A reflector holder designed to hold two fluorescent tubes was suspended from the ceiling. The containers were arranged on the room floor in a manner so that only one side and the top of each container were exposed to the desired light intensity. The walls of the room were covered with black paper to avoid any outside reflection of light. The three different light intensities investigated were 150 ft-c, 300 ft-c and 450 ft-c, measured at the necks of the containers. The distance of light source from containers was adjusted to obtain the desired light intensity. A Tektronix J16 digital photometer was used to measure the light intensity. Because of the physical limitations, no storage studies were conducted at 450 ft-c light intensity at 1.7°C storage.

At the beginning of an experiment, whole milk packaged for regular shipment was brought from a local dairy in regular gallon-sized paperboard containers. This prevented any influence of light prior to storage experiments. The product was then transferred into the experimental containers. Milk samples were assayed for riboflavin concentration after 0, 24, 48 and 72 hr storage. Sufficient containers were placed in storage to allow a new container to be sampled at each interval.

At each sampling storage interval, contents of the containers were well mixed before taking the required sample. Care was taken to minimize exposure to light during the transfer, handling and sampling operations. Simultaneous storage studies were conducted under dark for all containers.

Riboflavin was measured in duplicate sam-

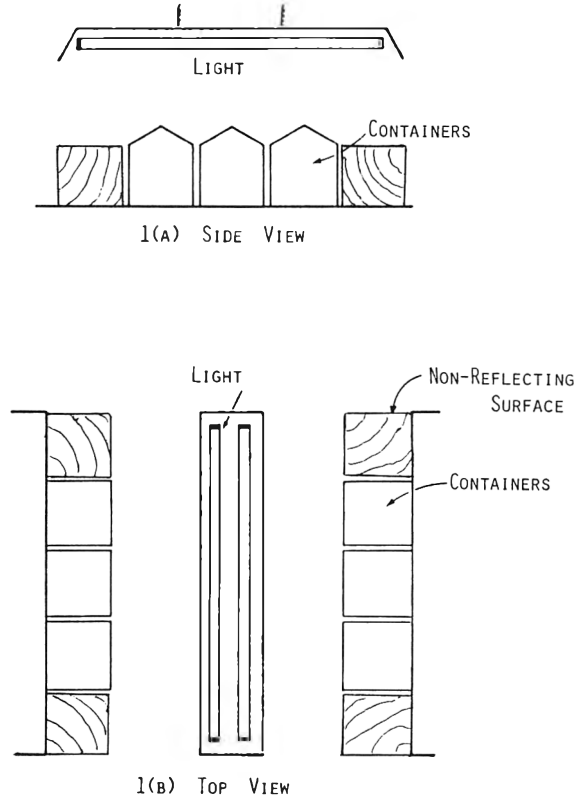

Fig. 1—Schematic layout of containers in storage.

ples by the continuous flow analysis procedure of Kirk (1974). This procedure is a modification of that published in *Methods of Vitamin Assay* (AOVC, 1966) and *Methods of Analysis* (AOAC, 1970) for continuous flow analysis.

Theory

The riboflavin degradation reaction was assumed to follow first-order rate kinetics:

$$\frac{d(C)}{dt} = -k(C) \qquad (1)$$

where (C) = concentration of riboflavin (mg/liter); t = time (hr); and k = first-order rate constant ($hour^{-1}$).

The percent loss of vitamin after 't' hours of storage was calculated from the rate constant (k) using the following expression:

$$\% \text{ loss} = [1 - \exp(-kt)]\,100 \qquad (2)$$

The reaction rate constant was assumed to depend upon temperature as described by the Arrhenius equation:

$$k = A \exp(-E/RT) \qquad (3)$$

where A = Pre-exponential frequency factor ($hour^{-1}$); E = Activation energy (calories/mole); R = Gas constant (1.98717 calories/mole K); and T = Absolute temperature (Kelvin).

A computer program, KINFIT, was used to fit the experimental data in Eq. (1) and (3). This program differs from the usual least-squares techniques, as it does not "linearize" the problem; however, it uses the numerical integration procedures to provide a fit to the desired differential equation (Dye and Nicely, 1971). The program is useful in plotting the data to the best fit and calculating standard deviations on the calculated parameters. The input of the variables (e.g., vitamin concentrations, time) is accompanied with their respective variances. This approach assists in accounting for the internal errors of vitamin assays and small variations in storage durations. The program is specifically written for chemical reactions and for evaluating kinetic parameters of these reactions.

RESULTS & DISCUSSION

A PLOT of experimental riboflavin concentration in milk as a function of storage time at 4.4°C in regular BMP and gold-pigmented BMP containers is shown in Figure 2. The decrease in vitamin concentration in these containers suggests an exponential degradation. Based on the earlier assumption that vitamin degradation is a first-order reaction, the measured data were fit into the model proposed in Eq. (1). This analysis was repeated for each storage condition. The various rate constants as computed using the KINFIT program are presented in Table 1.

The results in Table 1 indicate that the rate constants for vitamin degradation in milk stored in paperboard and gold-pigmented BMP containers are relatively small. This suggests negligible loss of vitamin. The standard deviations represent the data scatter of experimental analyses.

In addition, the rate constants were found to increase with storage temperatures for the same container under the same light intensity. This confirms that riboflavin photolysis is temperature-dependent.

The riboflavin losses in containers stored in dark were insignificant, and no further analysis was conducted under these conditions.

The rate of riboflavin degradation can be described by using another parameter

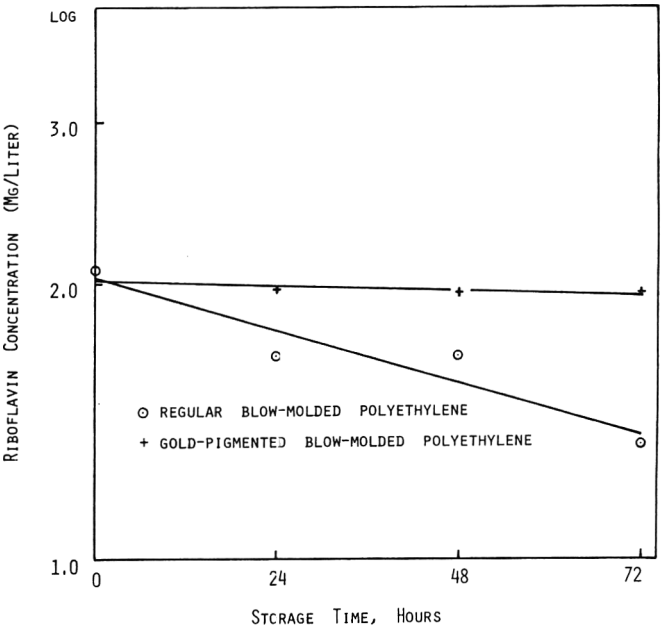

Fig. 2–Riboflavin degradation in milk stored at 4.4°C and 450 ft-c light intensity.

Table 1–Rate constants of riboflavin loss in milk

	10°C		4.4°C		1.7°C	
Container	Rate constant X 10^{-4} (hr^{-1})	Std dev X 10^{-4} (hr^{-1})	Rate constant X 10^{-4} (hr^{-1})	Std dev X 10^{-4} (hr^{-1})	Rate constant X 10^{-4} (hr^{-1})	Std dev X 10^{-4} (hr^{-1})
150 ft-c						
Glass	11.02	3.57	8.76	2.64	7.70	3.23
Blow-molded polyethylene (BMP)	10.54	2.72	5.99	0.34	4.41	1.00
Gold-pigmented BMP	3.93	4.12[a]	3.42	1.42	1.96	2.68[a]
Paperboard	2.72	2.36	3.89	0.81	1.02	0.36
300 ft-c						
Glass	32.22	4.38	24.25	9.3	20.89	2.12
Blow-molded polyethylene (BMP)	31.15	9.10	22.72	8.73	16.52	2.53
Gold-pigmented BMP	9.16	4.11	6.97	3.61	2.72	2.39
Paperboard	10.50	4.82	3.28	3.85[a]	0.62	2.59[a]
450 ft-c						
Glass	35.90	11.60	33.32	4.60	–	–
Blow-molded polyethylene (BMP)	52.51	13.0	34.17	8.30	–	–
Gold-pigmented BMP	5.52	2.64	3.16	5.47[a]	–	–
Paperboard	1.81	4.76[a]	4.14	5.80[a]	–	–

[a] These high standard deviations indicate data scatter with negligible loss of riboflavin.

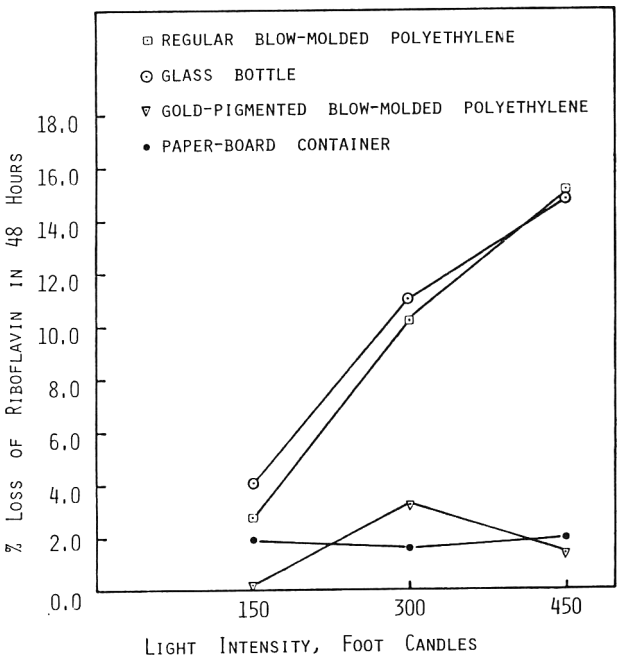

Fig. 3—Influence of light intensity on riboflavin degradation in milk stored at 4.4°C.

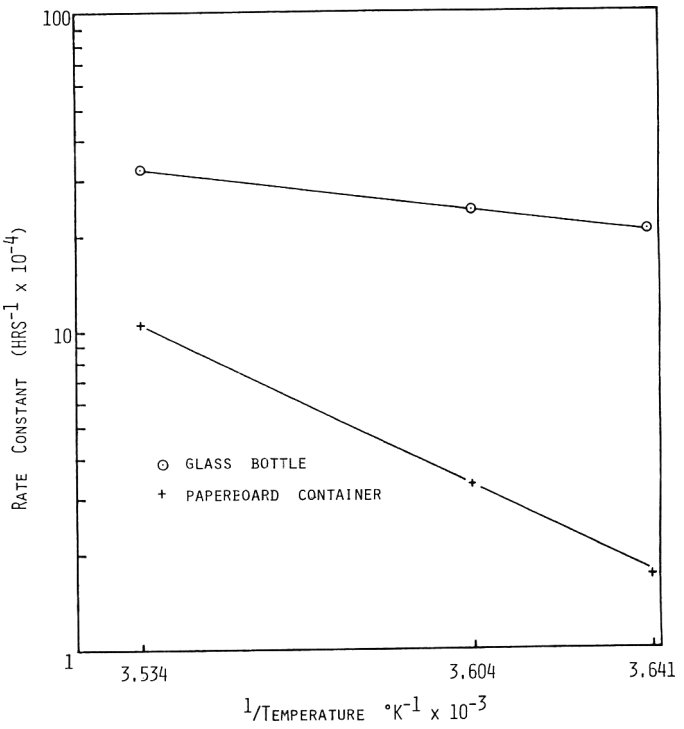

Fig. 4—Arrhenius rate plot of degradation rate of riboflavin as a function of 1/T°K.

computed from the rate constant; the percent loss of riboflavin in 48 hr storage, as calculated using Eq. (3). A plot of riboflavin loss versus light intensity at 4.4°C storage is shown in Figure 3. The results clearly indicate the increase in the vitamin destruction in milk stored in glass and regular BMP containers with increased light intensity. Glass and regular BMP containers also show the highest losses of riboflavin for each light intensity. The gold-pigmented BMP container was as effective as the paperboard container in providing maximum protection for riboflavin at each light intensity.

In order to calculate the activation energies, the rate constants were plotted versus the inverse of storage temperature (absolute) on semi-log coordinates. The slope of the straight line is directly related to the activation energy as shown in Figure 4. The KINFIT program was used to calculate the activation energies for various storage conditions. The results are tabulated in Table 2. The activation energy is lowest in glass and highest in the paperboard container. These results indicate the significant effect of temperature on riboflavin degradation in glass resulting in low activation energy. The activation energy can also be used in interpolating the rate constants at different storage temperatures.

The above discussion illustrates that the various kinetic parameters can be used to compare different containers. The kinetic analysis is particularly useful when the measured concentration is small and the experimental procedures may cause data scatter. By measuring the concentrations of a particular nutrient over a period of time, the parameters, such as rate constant and activation energies, can be calculated along with their respective standard deviations. These parameters are in turn useful in selecting the optimum storage conditions for the product.

CONCLUSIONS

1. The kinetic analysis indicates that riboflavin degradation in milk can be best described by assuming a first-order reaction.
2. The riboflavin loss increases significantly with an increase in storage temperature and incident light intensity in glass and regular blow-molded polyethylene containers.
3. The riboflavin loss after 48 hr of exposure under 300 ft-c light intensity and 4.4°C storage is approximately 11% in glass and regular blow-molded polyethylene containers, and only 3% in paperboard and gold-pigmented blow-molded polyethylene containers.
4. No significant loss of riboflavin was observed in dark while loss was controlled to low levels at 150 ft-c and 1.7°C storage in all containers.
5. Results obtained at 300 ft-c indicate that the magnitude of the activation energy is an indicator of the protective influence provided by the container.

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Table 2—Activation energies of riboflavin in milk stored under 300 ft-c in different containers

Container	Activation energy calories/mole	Std dev
Glass bottle	8032	15
Blow-molded polyethylene (BMP)	11878	1470
Gold-pigmented BMP	18555	9210
Paperboard	41223	9630

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MASS PRODUCTION OF Rhizopus oligosporus SPORES AND THEIR APPLICATION IN TEMPEH FERMENTATION

INTRODUCTION

TEMPEH is a popular Indonesian food made by fermenting soybeans with *Rhizopus*. In 1967, we (Hesseltine et al.) reported that tempeh-like products also could be made from wheat, rice, other cereal grains and various combinations of them. Solid, cake-like tempeh has a mild and pleasant flavor when fried in vegetable oil and, therefore, has the potential for use in a variety of high-protein snacks.

Tempeh fermentation is characterized by its simplicity and rapidity. The lack of a suitable inoculum, however, could be a hindrance since it is essential that the inoculum be pure and that spores have a high degree of immediate germinability. Without these conditions, the fermentation process is almost inoperable. Traditionally, small pieces of tempeh from a previous fermentation serve as inoculum. The fungus is then propagated mainly by means of fast-growing mycelia. This practice can lead to contamination by undesirable microorganisms, and the inability of mycelia to survive adverse temperatures and dehydration makes mycelia unsuitable for long-term preservation of their viability. In this country both Hesseltine et al. (1963) and Steinkraus et al. (1960) developed pure culture fermentation of tempeh. However, preparing agar media for mass production of spores is expensive and time-consuming. Steinkraus et al. (1965) used freeze-dried, 4-day fermented soybeans as inoculum. Because we experienced failure and uncertainties with a similar preparation, we undertook to develop a tempeh inoculum having a high, viable spore count that would maintain its viability for a long time with minimal attention.

METHODS & MATERIALS

Cultures

Rhizopus oligosporus NRRL 2710 was maintained on slants of potato-dextrose-agar (PDA) at 4°C. Before each experiment, the organism was transferred to another PDA slant and incubated at 28°C for 7 days. A spore suspension for inoculation was prepared by adding 3 ml of sterilized, distilled water to each slant and shaking the culture vigorously for 1 min.

Spore production

Solid state fermentations, consisting of such grains as pearled wheat, cracked soybeans, polished rice and wheat bran, were used to prepare spores. In each 300-ml Erlenmeyer flask, 10g of a specific grain and various amounts of water were mixed and allowed to stand at room temperature for 1 hr with frequent shaking. The cotton-plugged flasks were autoclaved at 120°C for 20 min and then cooled to room temperature. Each flask was inoculated with 0.1 ml of spore suspension (10^5 viable counts). An incubation temperature of 32°C was used because preliminary work indicated that the sporulation of *R. oligosporus* was less at 25°C than at 32°C.

Viable spore count

The viable spores and other propagules of the fermentation mass, before and after freeze drying, were estimated by plate count. 1g of thoroughly mixed fermentation mass was aseptically weighed and transferred to a sterilized Waring Blendor containing 99 ml of sterilized water. After blending 2 min at high speed, serial dilutions were made from this initial dilution (1:100). A 1-ml suspension from each dilution was mixed with 10 ml of plate count agar of 45°C (0.5% bactopeptone, 0.25% yeast extract, 0.1% dextrose and 1.5% agar) in a petri dish. After the dishes were held at 32°C for 20–24 hr, the colonies were counted.

Aerobic bacterial count

Aerobic bacterial counts were made by using plate count agar dishes containing 100 ppm Actidione®. Solutions of the antibiotic (10 mg/ml) were sterilized through Millipore® filters (0.45μ) and added to agar at 45°C. The dishes were held at 32°C for 3 days, and colonies were counted daily.

Tempeh fermentation

Cracked soybeans or pearled wheat were washed and soaked in water at room temperature for 30 min and boiled in excess water for 25 and 12 min, respectively, as described by Hesseltine et al. (1967). Freeze-dried spore preparations were mixed thoroughly with drain-dried, boiled grain. The inoculated grain was packed in petri dishes, trays or plastic tubing (5 × 15 cm) (Martinelli and Hesseltine, 1964) for incubation at 32°C for 18–22 hr.

Residue of water-extracted ground soybeans

Cracked soybeans were soaked in water at room temperature for 1 hr and washed free of hulls. The beans were wet ground in a Waring Blendor with additional water at a ratio of approximately 1:10 (w/v). The slurry was boiled for 15 min and filtered through a double-layered cheesecloth. The filtrate is usually referred to as soybean milk, from which tofu is made. The residue retained by the cheesecloth is the insoluble fraction.

RESULTS & DISCUSSION

Moisture requirement for sporulation

The moisture content of any substrate is of utmost importance in solid fermentation. When *R. oligosporus* was grown on various substrates for 4 days at 32°C, its growth and sporulation varied with the ratio of substrate to water. The viable spore counts of freeze-dried preparations made by growing *R. oligosporus* on four substrates–rice, pearled wheat, wheat bran and cracked soybeans–at three dif-

Table 1–Spore production of Rhizopus oligosporus grown on various substrates of three moisture levels

Substrate	Substrate:H_2O (w/v)	Substrate moisture (%)[a]	Viable spore count/g[b]
Rice	10:4	31	6.82
	10:6	40	8.03
	10:8	47	7.86
Wheat	10:4	34	6.70
	10:6	43	5.30
	10:8	50	5.30
Wheat bran	10:4	32	5.70
	10:6	40	6.95
	10:8	49	7.00
Soybeans	10:4	33	5.00
	10:6	39	5.70
	10:8	48	6.48

[a] Substrate moisture is determined by drying at 110°C for 24 hr.
[b] Counts are expressed as the logarithm of the numerical counts per g of freeze-dried preparation.

Table 2—Viable spore counts of Rhizopus oligosporus as affected by fermentation time, substrate and freeze drying

	Viable spore count/g[a]							
	Rice		Rice:wheat bran		Wheat		Wheat:wheat bran	
Day	B[b]	A[c]	B	A	B	A	B	A
4	9.05	8.01	9.21	7.82	8.01	6.91	8.94	8.25
5	9.08	7.85	9.33	7.86	9.08	7.16	9.11	7.68
6	8.48	7.21	8.64	7.10	8.24	6.97	8.64	7.15
7	8.19	7.52	8.54	7.59	7.93	7.31	8.19	7.45

Least significant difference = 0.49.

[a] Counts are expressed as the logarithm of the geometric mean counts per g of sample on dry basis.
[b] B = before freeze drying
[c] A = after freeze drying

Table 3—Effect of storage time and temperature on the viability of freeze-dried Rhizopus oligosporus spores prepared from three substrates

Time (month)	Temp (°C)	Viable spore count[a]		
		Rice	Rice:wheat bran	Wheat:wheat bran
0		7.32	7.56	7.00
2	4	7.18	7.58	7.08
	22	6.00	6.85	5.90
4	4	7.40	7.46	7.23
	22	6.85	7.00	5.90
6	4	7.36	7.49	7.04
	22	6.60	7.08	5.95

[a] Counts are expressed as the logarithm of the numerical counts per g of freeze-dried preparation.

ferent moisture levels for 4 days are recorded in Table 1. The fungus sporulated abundantly on rice, wheat bran and soybeans at a moisture level of 40% as well as 50%, but not at 30%. On the other hand, a 34% moisture level provided the best condition for sporulation of *R. oligosporus* on wheat; sporulation decreased as the moisture level increased.

Wheat bran, reported as a good substrate for spore production by *Aspergillus ochraceus* (Singh et al., 1968) and by *Hemispora stellata* (Sala and Burgos, 1972), also is a good substrate for sporulation of *R. oligosporus*. When the spore preparation made from wheat bran was used as inoculum for tempeh fermentation, the distinct dark-brown specks of wheat bran gave an unfavorable appearance to the final product. After extended fermentation of soybeans by *R. oligosporus*, an unpleasant odor often resulted, perhaps due to their high-protein content. Therefore, soybeans and wheat bran alone were considered undesirable for making tempeh inoculum.

Interactions of fermentation time, substrate and freeze drying on viable spore counts

R. oligosporus was grown for 4 – 7 days at 32°C on four substrates: rice:water (10:6); rice:wheat bran:water (8:2:6); wheat:water (10:4); and wheat:wheat bran:water (8:2:6). On a dry basis, the viable spore counts per g preparation ranged from 6 to 257 X 10^7 before freeze drying and from 4 to 400 X 10^6 after freeze drying. Before freeze drying, the sample counts actually consisted of spores and other propagules, such as mycelial fragments. Therefore, low sample counts were expected after freeze drying. Not only do the mycelia succumb to adverse temperatures, but the percentage of spore germination is also affected adversely by freezing.

Data in Table 2 indicate the geometric mean spore counts from duplicate flasks as affected by fermentation time, substrate and freeze drying. As expected, sample counts taken before freeze drying were significantly higher than those taken after freeze drying. Analysis of variance indicated no significant interactions of freeze drying and substrates. Among the substrates tested for spore production by *R. oligosporus*, wheat was the poorest. The other three showed no differences.

Generally, adequate aeration is one of the environmental factors necessary for spore formation by *R. oligosporus*. The stickiness of the wheat substrate might have created an anaerobic condition unfavorable for sporulation. Attempts were made to improve the texture of wheat substrate by adding various amounts of wheat bran. As indicated in Table 2, a mixture of wheat and wheat bran (4:1) was a better substrate, on the one hand,

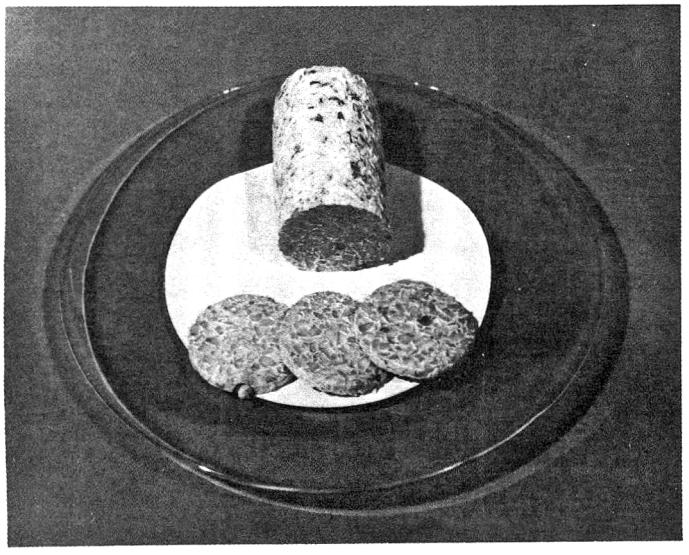

Fig. 1—Tempeh made from preinoculated soybeans after storing in a deep freezer for 2 months.

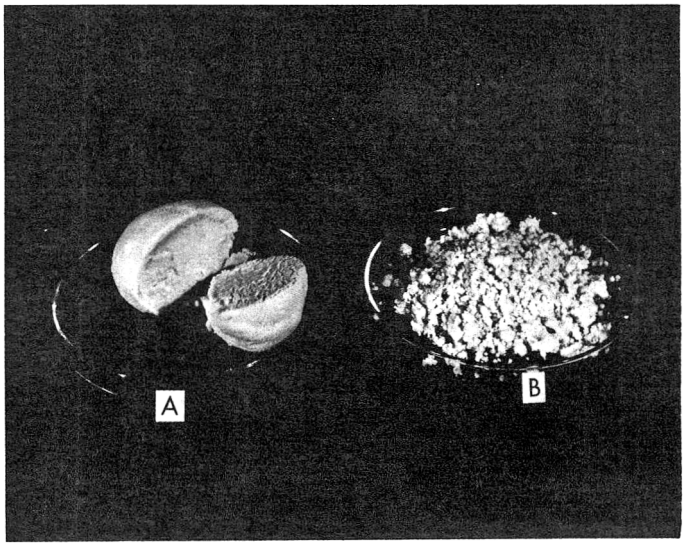

Fig. 2—Residue from water-extracted ground soybeans: (A) freshly prepared; (B) after drying at 100°C.

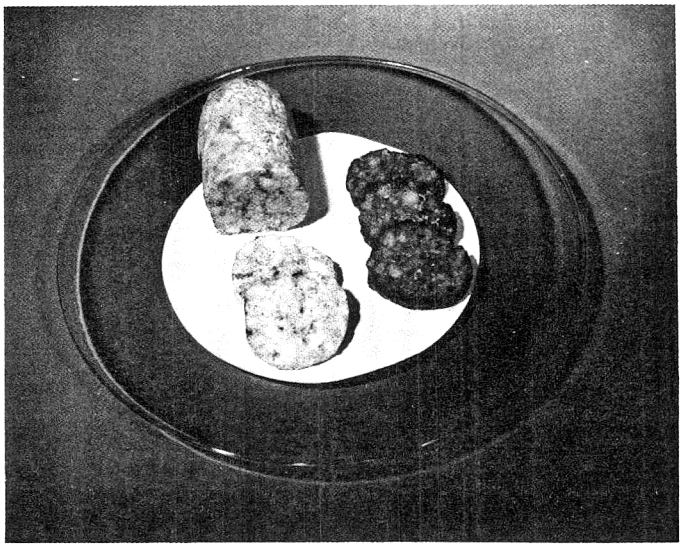

Fig. 3—Tempeh made from residue of water-extracted soybeans: left, fresh; right, cooked.

for spore formation by *R. oligosporus* than wheat alone. The addition of wheat bran to rice, on the other hand, had no effect on spore production.

Preliminary data indicated that spore production was much less after 3 days of incubation than after 4 days. Results in Table 2 showed adequate incubation time was from 4–5 days.

Based on these results, we suggest making *R. oligosporus* preparation by fermenting either rice, rice:wheat bran (4:1) or wheat:wheat bran (4:1) at a substrate to water ratio of 10:6 for 4–5 days at 32°C. The fermentation mass was then immediately freeze dried and ground into fine powder.

Storage stability

The freeze-dried and ground spore preparations were kept in closed plastic bags at 4°C or at room temperature (22°C) for 6 months. Their viable spore counts are summarized in Table 3. When the preparations were kept at 4°C for 6 months, the spore counts showed typical experimental variations and were comparable to their original counts; whereas, at room temperature, a significant decrease in viability of all three preparations was noted after 2 months. Thereafter, no further decrease was observed.

The possible contamination of the spore preparation by aerobic bacteria was checked. Bacterial contamination was not found to be a problem either during the process of fermentation or in storage.

Tempeh made with freeze-dried spores

To determine the amount of inoculum required to make satisfactory tempeh from either soybeans or wheat, various amounts of spore preparations having 10×10^6 viable counts per g were added to 100g of cooked grain. The fermentation was carried out in petri dishes. When the level of inoculum varied from 0.01–0.15g (1×10^5 to 1.5×10^6 spores), the time required to complete fermentation ranged from 22 hr to less than 17 hr. We suggest using 1×10^6 spores per 100g of cooked beans or wheat, because fermentation time becomes too critical if the amounts of inoculum are larger. On the other hand, too small an amount of inoculum provides a chance for contaminating bacteria to grow. At the inoculum level of 1×10^6 spores per 100g cooked soybeans, good tempeh was also made by tray and package fermentations as described by Martinelli and Hesseltine (1964).

Inoculated soybeans were packed in plastic tubing (5 × 15 cm) and stored in a freezer at −15°C to defer fermentation until the tempeh was needed. After 2 months of storage, the bags were perforated with holes having a diameter of about 0.6 mm and a distance of about 0.5 cm by a small needle to allow aeration for mold growth and were incubated at 32°C. Good tempeh (Fig. 1) was made after an incubation time (20–22 hr) no greater than that required by freshly inoculated beans. Thus, preinoculated beans can be packaged, stored in a freezer and sold to be taken home and allowed to ferment in a warm place.

Tempeh made from residue of water-extracted ground soybeans

The residue from making soybean milk and tofu, two main food products derived from water extraction of soybeans, has been considered a waste. Therefore, attempts were made to develop a palatable product from this residue by *R. oligosporus* fermentation. On a dry basis, the residue of water-extracted soybeans contains 32% protein, as determined by Kjeldahl digestion, and 20% oil as determined by ether extraction. Its moisture content is usually greater than 90%, so that the texture of the residue is too mashy to provide good growth for *R. oligosporus*. When moisture is reduced to less than 80% by drying at 100°C, this fraction appeared crumbly (Fig. 2) and was suitable for fermentation. After the fraction was inoculated with *R. oligosporus* spores, fermentation (Fig. 3) was completed after 20 hr at 32°C. Tempeh made from the residue of water-extracted soybeans has a texture and flavor similar to French-fried potatoes after deep-frying.

Hackler et al. (1963) reported that the water-insoluble fraction contains the highest quality protein, as measured by rat growth and protein efficiency ratio, among several soybean fractions studied: full-fat soybean flour, water-extract of soybeans, acid-precipitated curd and whey protein. Therefore, tempeh made from the residue of water-extracted soybeans is nutritious, as well as tasty.

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REMOVAL OF THRIPS DURING ASPARAGUS WASHING

INTRODUCTION

SEVERAL SPECIES of thrips are found on asparagus in New Jersey. Comegys and Schmitt (1957) reported seven species: *Thrips tabaci, Frankliniella fusca, Frankliniella tenuicornis, Limothrips cerealium, Aeolothrips fasciatus, Aeolothrips bicolor* and *Phlaeothrips karnyi* complex. During the harvesting season thrips apparently migrate to asparagus from surrounding vegetation. Truck loads of asparagus can be rejected and processing operations can be curtailed when asparagus become highly infested.

The objective of this research was to determine the optimum washing conditions for removing thrips from asparagus. The National Canners Association (Mercer et al., 1960) reported on the use of warm detergent baths for washing asparagus. Insect counts were found to be lower for detergent-washed asparagus. A foam cup of medium tenacity on the wash tank, from the use of foaming detergents, was reported to trap insects removed from the tank on the elevator or discharged over the side of the tank. Detergents of the alkyl aryl sulfonate type were being used in these evaluations. No actual data on thrips removal was provided, however. This research as well as the National Canners Association 1962 Report (Mercer, 1962) studied detergent washing of asparagus primarily for soil removal and the removal of bacterial organisms carried on the soil that can cause bacterial spoilage in canned asparagus. Soil removal was enhanced by increased soak temperatures (43–93°C) as well as by increased rinse spray pressures (20–40 psi) and low-foaming detergents were superior to the high foam types evaluated. Our experimentation for thrips removal evaluated soak water temperatures, rinse spray pressures, a low-foaming wetting agent and the addition of pyrethrins in the soak water to act as an irritant to thrips.

EXPERIMENTAL

THRIP contaminated asparagus spears were transported from the field to the Food Science Department and stored at approximately 22°C. The day following harvest each 20-lb box of asparagus was split. The asparagus spears for a given replicate were mixed, butted to give a 5-in. spear, and divided into 2-lb batches for washing. The first replicate was washed 1 day after harvest and the second, 2 days after harvest.

Asparagus spears were washed for 2 min in an A.K. Robins Co. Blancher-Scaler Washer equipped with steam for heating the water and compressed air for agitation (catalog 1100 Fig. 37305). From the washer the asparagus was dumped onto a 6-ft roller conveyor (catalog 110 Fig. 115-5, A.K. Robins Co.). The 1-3/8 in. diam rollers were spaced 1/8 in. apart and moved at a rate of 18 ft/min. A spray manifold 8 in. above the roller conveyor provided a spray rinse. Different spray pressures were obtained by means of a variable speed pump connected to the spray manifold with a flexible high-pressure hose. The spray manifold contained six rows of nozzles: two rows of flat jet nozzles (3/8 P 3530, Spraying Systems Co.) were followed by one row of full jet nozzles (3/8 GG15, Spraying Systems Co.) and the sequence was duplicated.

Two samples of washed asparagus, each weighing 3/4 lb, were taken from each treatment, placed in cans and butted; hot 2% brine solution was added, the cans sealed and placed in a pressure cooker at 118°C for 25 min.

Thrip counts were determined in the following manner: first the brine from an opened can was passed through filter paper and the trapped thrips counted. Secondly, the asparagus spears were macerated in a flat pan and then processed through a trap flask for insect and fragment counts, as described in AOAC (1965). The total numbers obtained from both procedures were reported.

The data from all experiments were transformed to square roots [transformed count = square root (thrips count + 1)] and analyzed statistically using the analysis of variance appropriate for the factorial design. Significant effects were determined at 0.05 probability

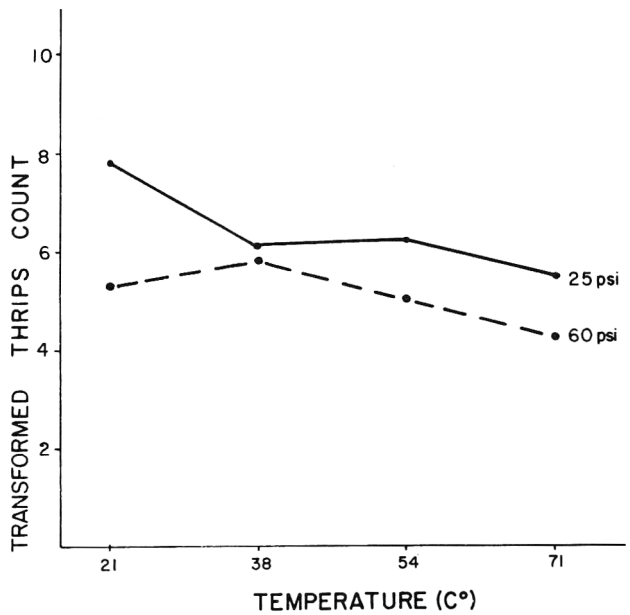

Fig. 1—Thrips removal from asparagus (average of two replicates) after a 2 min soak followed by spray rinsing.

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level. In both experiments the experimental variation (treatment to treatment) of the transformed counts was larger than the sampling variation (can to can). The standard deviation (transformed counts) of the experimental variation was 0.74 units in the first experiment and 1.54 units in the second experiment.

RESULTS

THE FIRST EXPERIMENT evaluated the effects of washing temperature (21, 38, 54 and 71°C), spray pressure (25, 60 psi), and wetting agent (0, 0.025% sodium dodecylbenzene sulfonate) on thrips removal using a 4 X 2 X 2 factorial design with two replicates.

The analysis indicated that temperature, pressure and the temperature X pressure interaction (Fig. 1) had a significant effect on thrips removal. The wet-

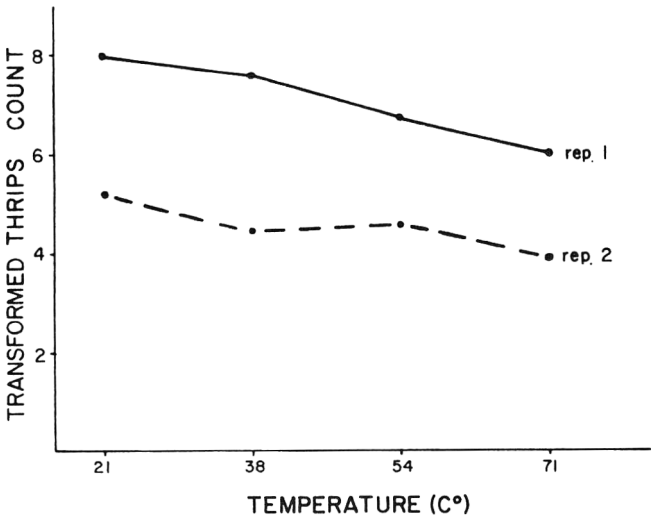

Fig. 2—Thrips removal from asparagus (average of two rinse pressures) after a 2 min soak followed by spray rinsing.

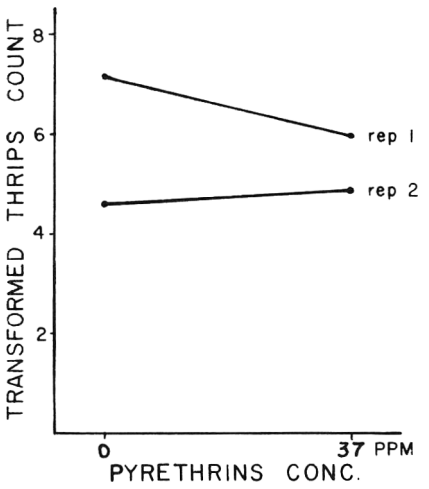

Fig. 3—Thrips removal from asparagus after a 2 min soak with and without 37 ppm pyrethrins followed by spray rinsing.

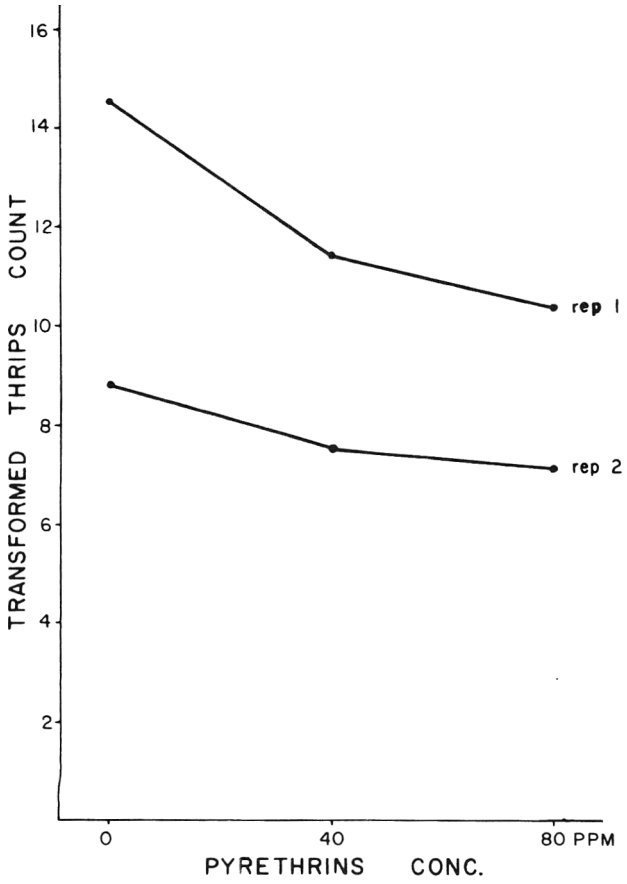

Fig. 4—Thrips removal from asparagus (average of four soak temperatures, two replicates and three spray pressures) after a 2 min soak followed by spray rinsing.

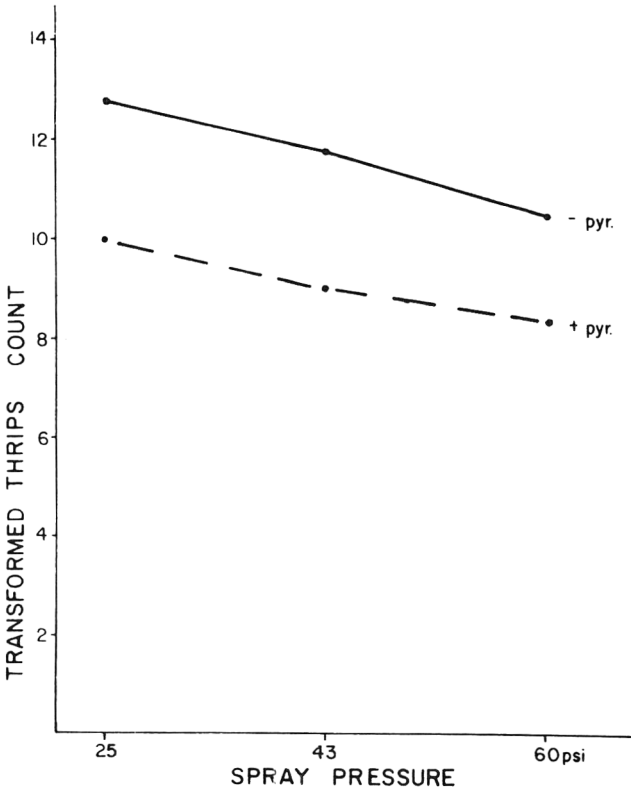

Fig. 5—Thrips removal from asparagus (average of four soak temperatures and two replicates) after a 2 min soak with and without pyrethrins (average of 40 and 80 ppm) followed by spray rinsing.

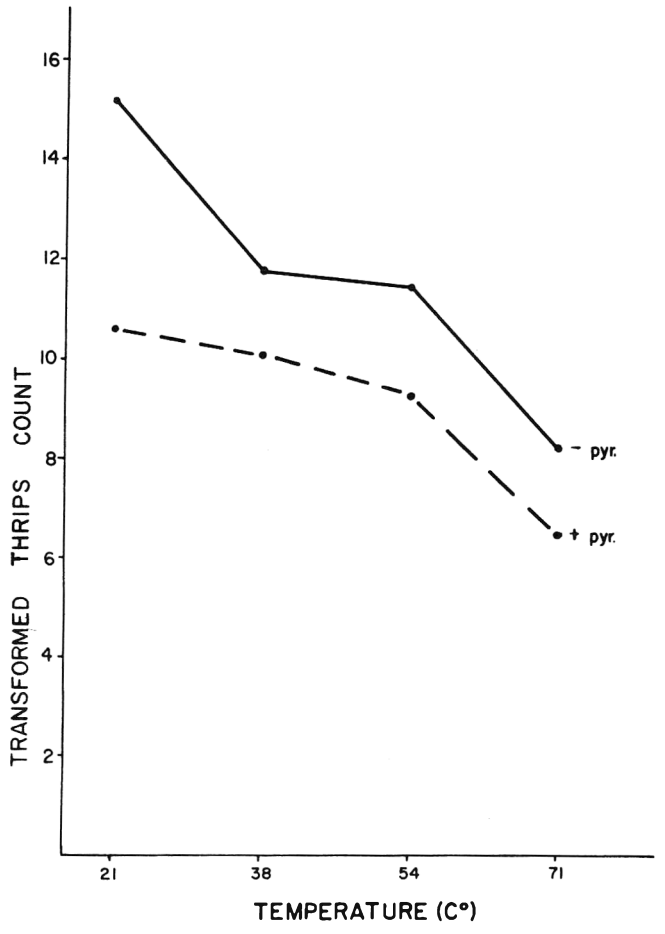

Fig. 6—Thrips removal from asparagus (average of three spray pressures and two replicates) after a 2 min soak with and without pyrethrins (average of 40 and 80 ppm) followed by spray rinsing.

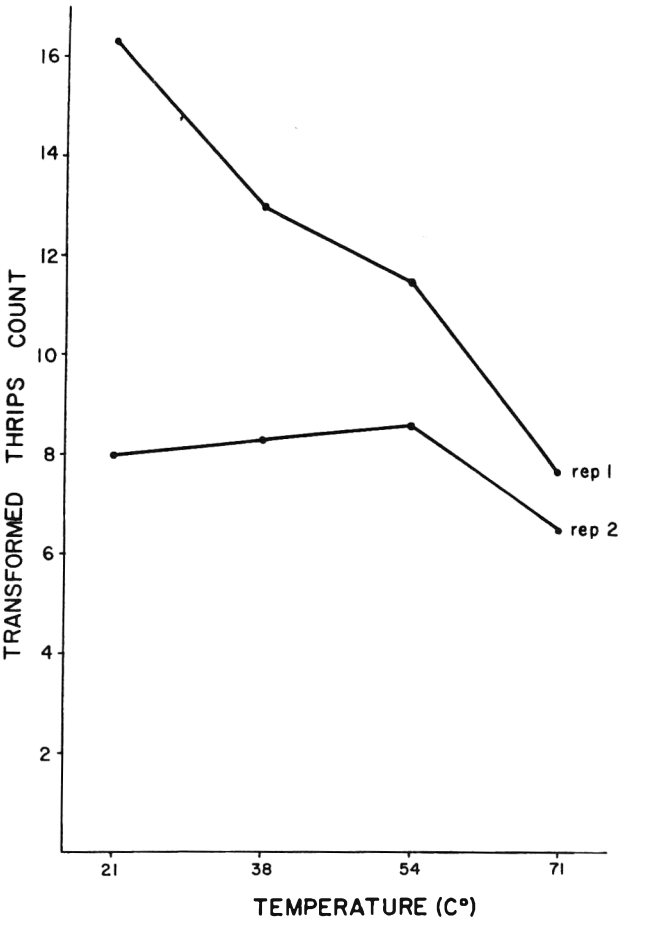

Fig. 7—Thrips removal from asparagus (average of three spray pressures) after a 2 min soak with and without pyrethrins (average of 40 and 80 ppm) followed by spray rinsing.

ting agent did not have a significant effect. Increasing temperature and pressure increased thrips removal; however, the pressure effect was larger than the temperature effect and varied depending upon the temperature.

As was stated in the Experimental section, the first replicate was washed 1 day after harvest and the second, 2 days after harvest. Thrips were observed flying around the stored asparagus boxes when replicate two was transferred to the pilot plant. The "storage effect" shown in Figure 2 evidenced a decided reduction in thrips count (replicate two) at all temperature levels.

A separate test in the first experiment evaluated the combined effect of 37 ppm pyrethrins and 410 ppm piperonyl butoxide added to the wash water (Fairfield Chemicals Pyrenone containing 7.5% pyrethrins, 84.0% piperonyl butoxide; 12.5% triton X-155 was added to the pyrenone to emulsify the oil). The wash tank also contained the wetting agent 0.025% sodium dodecylbenzene sulfonate. Analysis of the data comparing the pyrethrins treatment with the control containing a wetting agent indicated that pyrethrins produced a significant increase in thrips removal in replicate 1, but the effect was not present in replicate two (Fig. 3).

In the experiment conducted the following year the effects of washing temperature (21, 38, 54 and 71°C), spray pressure (25, 43, 60 psi), and pyrethrins (0, 40, 80 ppm) on thrips removal were determined using a 4 X 3 X 3 factorial design with two replicates. [Pyrenone concentrate 778 (Fairfield Chemicals) containing 6% pyrethrins and 60% piperonyl-1-butoxide.]

The analysis of the data indicated significant main effects due to washing temperature, spray pressure and pyrethrins; significant interactions between temperature and pyrethrins, temperature and replicates, and pyrethrins and replicates; and, a significant difference between the replicates. In general, increasing temperature (Fig. 6 and 7), pressure (Fig. 5), and pyrethrins concentration (Fig. 4 and 5) significantly increased thrips removal. The pressure effect was independent of temperature, pyrethrins concentration and replication; however, the temperature (Fig. 7) and pyrethrins (Fig. 4) effects were larger in replicate one than in replicate two and the pyrethrins effect was larger at 21° than at 38, 54 and 71°C (Fig. 6). As in the first experiment, there were significantly fewer thrips in replicate two than in replicate one.

The experiments indicate that increases in washing temperature, spray pressure and pyrethrins concentration will produce significant increase in thrips removal.

DISCUSSION

HOT WATER SOAKS and high pressure sprays, reported to aid in soil removal from asparagus (Mercer, 1962), have been found to enhance the removal of thrips. Presumably the hot water opens the asparagus head and makes the spear more pliable; the pressure sprays provide the physical forces for removing both soil and thrips. A low-foaming wetting agent (so-

dium dodecylbenzene sulfonate), which has been reported effective for soil removal (Mercer, 1962), did not aid in thrips removal. The reduction of soak water surface tension with 0.025% sodium dodecylbenzene sulfonate was found to be approximately 50% (from 73 dynes/cm to 36.5 dynes/cm). Pyrethrins added to the soak water at low concentrations significantly enhanced thrips removal. Pyrethrins are believed to act as an irritant to thrips, causing them to move from under the asparagus bracts and head.

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REFRIGERATED BULK STORAGE OF CRANBERRY PUREE

INTRODUCTION

THE CONVERSION of fruits and vegetables into purees for bulk storage and subsequent processing is a recent innovation of the food industry in this country. The concept of bulk storage is not new. There are many examples of bulk holding techniques that utilize control of a "processed product environment" by chemical modification, temperature modification, dehydration, pasteurization or sterilization and subsequent protection from recontamination (Pearl, 1971). There are many economic and practical advantages that make bulk storage processing more desirable than traditional methods of immediate processing (Sullivan, 1971).

The conversion of bananas (Brekke et al., 1969), guavas (Brekke et al., 1970), and tomatoes (Nelson, 1971) into stable purees for bulk storage has been reported. The successful preservation of these fruit purees was dependent on the addition of fungistatic agents, such as sorbate salts (Brekke et al., 1969). Clague and Fellers (1934) and Fellers and Esselen (1955) failed to find a relationship between the natural fungistatic agents in cranberries, benzoic and quinic acids, and their storage stability.

Estimates of conditions, times and temperatures required for enzyme inactivation in fruit purees has been summarized by Dimick et al. (1951). Oxidative and pectic enzyme systems of the McFarlin cranberry have been identified and characterized by Chan and Yang (1971) and Arakji and Yang (1969), respectively.

Quality assurance of purees during bulk storage is necessary. Saravacos (1968, 1970) concluded that the consistency of peach, apricot and pear purees was a function of concentration, size and shape of the suspended solids in the puree. Color measurements of cranberries and cranberry products have been reviewed by Francis and Clydesdale (1970). Objective measurements of consistency and color of peach purees have been correlated with subjective taste panel evaluations (Wilson et al., 1957).

Because cranberries are richer in pectin than other fruits (Smith, 1959), the gel-forming capability of cranberry pectin (Yueh, 1957; Chawan, 1965), and the production of cranberry sauces is economically important. Servadio and Francis (1963) and Zuckerman et al. (1966) reported a significant correlation between the color and pigment of fresh cranberries and the color of the cranberry sauces produced. Rank (1963) related sensory evaluations of cranberry sauces to the gel strength, weep, sugar concentration and levels of acidity.

It is apparent that a major bottleneck in the cranberry industry is in the complete processing of cranberries. A method of efficiently storing the fruit until the rush of the harvest season is past would increase the efficiency and capacity of a processing facility. The purpose of this study was to determine the practicability of converting cranberries into a stable puree for long term bulk storage and subsequently preparing a quality cranberry product from this puree.

MATERIALS & METHODS

SEARLES CULTIVAR cranberries (*Vacinnium macrocarpon*, Ait.) were steam heated to 88°C in a thermoscrew and comminuted through a 0.30 in. screen to provide a conventional puree used in the production of strained cranberry sauce. The cranberry puree was cooled at 24°C in a large water-cooled vat over a period of 4 hr, divided into 10 gal stainless steel "bulk tanks" and transported from a cranberry processing plant to the laboratory. Several No. 10 (603 × 700) tins of the same cranberry puree were hot-packed, sealed, water cooled and maintained as a check along with the bulk storage samples.

The cranberry puree in the 10 gal containers was held under nine storage conditions (Table 1). Atmospheres of carbon dioxide, air, nitrogen and dichlorodifluoromethane (Freon 12) were maintained at 5 psi guage pressure. One storage tank was maintained with a partial vacuum of approximately 5 psi absolute.

Table 1—Bulk storage conditions of cranberry puree

Condition	Temp (°C)
No. 10 tins (control)	4
No treatment (control)	4
0.1% Sorbic acid (by weight)	4
0.28% antioxidant [Avenex] (by weight)	4
Partial vacuum (ca. 5 psi absolute)	4
5 psi carbon dioxide	4
5 psi dichlorodifluoromethane (Freon 12)	4
5 psi compressed air	4
5 psi nitrogen	4
5 psi nitrogen	21

Subjective observations of mold and yeast growth were recorded weekly as samples were removed from the surface of the cranberry puree in the bulk container. The soluble and total solids concentrations, pH and titratable acidity of each sample were determined. Color difference was determined by placing cranberry puree in a plexiglass container and determining color difference with a color difference meter (Hunterlab Model D25) standardized with the white standard (Hunterlab D25-1300).

Consistencies of the cranberry purees were determined with a Brookfield Viscometer (Model RV) at 4°C. The No. 5 spindle and 5 rpm were arbitrarily selected for these measurements. The gel-forming capabilities of the cranberry purees were determined using the method of Weckel and Swanson (1972).

After 22 wk of refrigerated bulk storage, strained cranberry sauces were prepared from the remaining cranberry puree. The cranberry sauces were formulated to contain 40% puree, 38% sugar and 22% water. The gel strengths of these sauces were determined with a penetrometer (von Elbe et al., 1967). The color differences were evaluated with the color difference meter. The firmness, color and flavor of the cranberry sauces were evaluated by a taste panel. A 2-oz portion of each sauce was presented for evaluation on a ranking intensity ballot of 1 (poorest) to 9 (best) with selected adjectives to define gel strength, color and flavor extremes.

Regression coefficients between characteristic properties of the purees and time of storage were computed. Correlation analysis of subjective and objective evaluations of the cranberry sauces were also computed (Steel and Torrie, 1960).

RESULTS & DISCUSSION

UNTREATED cranberry puree supported mold and yeast growth after 3 wk of refrigerated bulk storage. Mold growth was prominent on the surface of cranberry puree under aerobic atmospheres, but was prevented, as was yeast growth, with the addition of 0.1% sorbic acid or aseptically packing the cranberry puree at 88°C. Anaerobic atmospheres (carbon dioxide, nitrogen and dichlorodifluoromethane [Freon 12]) prevented the growth of molds, but did not prevent the growth of yeasts. Anaerobic conditions were not detrimental to the composition of the cranberry puree.

The mean concentrations of soluble and total solids of the cranberry purees changed significantly during the 22 wk of storage at 4°C (Table 2). At the conclusion of bulk storage, the concentrations

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Table 2—Mean properties of cranberry purees during 22 wk of bulk storage at 4°C

	Weeks of bulk storage					
	1	9	22	Means	Ranges	St dev
Properties						
Soluble solids (%)	7.1	7.1	6.5	7.0**	5.5– 7.9	0.20
Total solids (%)	9.06	8.65	8.10	8.62**	6.57– 9.93	0.33
pH	2.76	2.70	2.67	2.67	2.64– 2.78	0.03
TA (% citric acid)	1.90	1.93	1.94	1.93	1.80– 2.16	0.01
Color difference[a]						
L	18.6	19.1	19.5	19.1**	17.8–20.6	0.46
a	26.2	29.6	29.1	28.9	25.3–33.5	1.60
b	5.7	5.6	5.7	5.5	4.4– 6.5	0.22
a/b	4.5	5.2	5.1	5.1*	4.4– 6.2	0.38
ΔE	79.9	80.6	80.2	80.4	78.2–81.4	0.36
Consistency (1000 cp)	46.1	47.0	46.0	46.5	13.2–58.8	1.4
Gel factor	34	35	37	36**	33 –40	1.0

[a] Color differences determined from white standard (Hunterlab D25-1300)
* $P < 0.05$
** $P < 0.01$

of soluble and total solids of samples representative of the remaining cranberry purees were analyzed and solids gradients were observed. The significant change in solids concentrations was a result of unrepresentative samples taken from the surface of the cranberry puree in storage, and not a result of a decrease in solids concentration throughout the purees. The introduction of agitation to prevent this natural formation of solids gradients may be necessary.

Cranberry puree was shown to be a pseudoplastic non-Newtonian fluid similar to other fruit purees described by Saravacos (1970). The consistency of cranberry puree did not change significantly during long term refrigerated bulk storage. The cranberry puree was consistently pourable and pumpable at all temperatures at 4°C or above.

The color difference date from cranberry purees were difficult to interpret (Table 2). The experimental significance of the lightness (L) values was attributed to diffusion of solids and dilution of color from the surface of cranberry puree during storage and the apparent mold and yeast growth at the surface. The redness (a) and yellowness (b) experimental means were not significant, indicating little change in the reflectance of these wavelengths. Derived color difference values such as a/b, Lb/a, and ΔE often present another perspective on color changes taking place. Significance of a/b experimental factors was the result of slight increases in redness (a) and slight decreases in yellowness (b) during the course of this study. Derived color difference values (Lb/a and ΔE) were not advantageous. Color differences were not visible or significant to the practical use of cranberry puree after prolonged bulk storage.

The pH and titratable acidities of the cranberry purees did not change significantly during storage. The development of gel factor index was a means of establishing pectin capability of cranberry pectin in a crude but practical preparation (Chawan, 1965). The gel factor indices of cranberry purees stored for 22 wk at 4°C indicated a significant change in the capability of the puree pectic substances to form a satisfactory gel. The treatments of the cranberry purees were shown to increase the capacity of cranberry pectin in situ to carry sugar, rather than to decrease this capacity as determined in isolated pectin systems by Meyers and Baker (1934).

Analysis of the strained cranberry sauces prepared at the termination of 22 wk of storage at 4°C indicated that gel strength was affected by storage conditions. Gel strengths as measured with a disc penetrometer varied from fluid (25.3g) to very firm (83.4g). The gel strengths of the strained cranberry sauces made from the purees were weaker than the gel strength of commercial cranberry sauce (90.3g). Penetrometer gel strengths were significantly correlated ($r = 0.94$) with gel strengths as determined by the taste panel.

The color differences among the cranberry sauces were very small. The objective color difference values, L, a, b, a/b, Lb/a, and ΔE, correlated poorly with subjective color evaluations by the taste panel. The mean flavor scores of the cranberry sauces ranked for intensity ranged from 3.57–5.77 on the 9.00 scale. The detecion of 'yeasty' and 'stored' flavors were associated with cranberry sauces made from purees which had supported large yeast or mold populations. Overall, the flavor scores indicate the cranberry sauces produced from cranberry puree stored in bulk containers at 4°C were not undesirable.

Cooperative preliminary investigations of bulk aspetic storage of cranberry puree at 4°C and 27°C were undertaken at Purdue University (Nelson, 1971). These experiments suggest a complete absence of microbiological problems and hold the promise of economically sound year-round processing of cranberry products of excellent quality. More recent studies (Weckel, 1974) indicate that cranberry puree may be held in a bulk storage tank for up to 6 months at 4°C with a flowing overlay of gaseous carbon dioxide. Inactivation of the pectinase enzyme systems in cranberry puree prior to bulk storage was found to be very important. With complete inactivation of these enzymes, the bulk storage of cranberry puree appears to be a practical solution to long-term preservation of cranberry puree for subsequent processing.

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ASCORBIC ACID RETENTION IN ORANGE JUICE AS RELATED TO CONTAINER TYPE

INTRODUCTION

THE CITRUS processing industry has traditionally been concerned with preserving natural nutritional qualities of their products, particularly ascorbic acid (Vitamin C). Many studies have been made (see below) on relationships between container, storage conditions and ascorbic acid (AA). Changes have developed in products, processes, containers and the materials and techniques used to fabricate them. There is need to study AA retention, as influenced by these new factors, to augment preceding studies.

Most early studies of single-strength orange juice (SSOJ) in tin-lined cans or glass bottles generally showed that up to 75% or more AA was retained after 1 yr at about 26.7°C or lower. SSOJ kept frozen in tin-lined cans at −17.8°C showed no change after 1 yr (Nelson and Mottern, 1933). Retention of AA in tin was slightly better than in glass, 80% or more after 6 months at 26.7°C (Moore et al., 1944). Pasteurized SSOJ in glass for 1 yr retained 87% AA at 4.4°C but only 68% at 26.7°C (Curl and Veldhuis, 1947). AA retention in frozen concentrated orange juice (FCOJ) in tin cans was 90% or greater after 1 yr, at 4.4°C or below, regardless of headspace atmosphere, concentration of product, or preliminary heat treatments (Curl et al., 1946). Curl (1947) also reported that loss of AA increased with temperature and concentration from 4.4°C to 48.9°C and from 13° to 71° Brix, respectively. Retention at 4.4°C ranged from 99% for SSOJ to 93% for 71° Brix concentrate and at 26.7°C was reduced to 70% and 6%, respectively, after 1 yr. DuBois and Kew, 1951, found FCOJ stored in tin-lined cans for 11 months at −28.9°C to 23.9°C had very high (95% or more) retention of AA. Heat treatment had little effect (McColloch et al., 1957), and samples stored from −12.2°C to 15.6°C) for 1 yr, to simulate conditions in some warehouses, retained 95% AA.

Changes in processes and packaging materials made it advisable to reevaluate AA retention in citrus products. A principle process for SSOJ uses the "hot-filled" procedure. Hot juice is poured into the preheated container, closed and cooled under water sprays, in effect, accomplishing a heat sterilization of package and product. The other widely used SSOJ process is "aseptic packaging" or "chill-pack" where the product is poured into the container at 4.4°C or lower, the package and closer having been previously sterilized with a chemical. Recent changes in packaging have included use of glass, polyethylene, polystyrene, cardboard, plastic and foil laminates as well as aluminum and tin-coated steel cans for various citrus products. There was also the question of whether AA retention is affected by the type container used for storing reconstituted orange juice in the home, and by in-home storage time before use. This is a report of a study to relate AA retention in orange juices and concentrates to these changing factors.

Although in these studies a slight amount of the AA may have been oxidized to dehydroascorbic acid (DHA – a biologically active vitamin), because the amounts were not likely to be large, the determination of DHA was not included. The relative effects of packaging and processing, would probably be similar on DHA since it too is subject to oxidation.

EXPERIMENTAL

Analytical method

All AA analyses were carried out using the standard AOAC method (1965), consisting of titration with a previously standardized 2,6-dichloroindophenol solution. All analyses were carried out on three to five replicates.

Single-strength "chill pack" orange juice, pasteurized

All juices and packaging materials in this part of the study were intended for commercial refrigerated handling. Packaging materials were glass, rigid polyethylene, high-impact polystyrene and waxed cardboard. Polyethylene bottles (64 fl oz) had sidewalls that average 0.024 in. thick. Polystyrene bottles (4 oz) had 0.012 in. sidewalls. Wax or plastic-lined cardboard cartons (64 oz) were similar to those used for milk.

Glass bottles (7 oz) were aseptically filled with juice (75% Hamlin, 25% Valencia) which had been held at −31.7°C since the previous season (about 6 months). The mixed juices had been pasteurized and pre-cooled before filling and crown-capping at a citrus processing plant. They were brought to the laboratory, held overnight at 4.4°C and assayed (9 bottles) for initial AA content. The samples were then divided and stored at 4.4, 10, 15.6 and 26.7°C. Glass bottled samples could not be stored at −6.7 and 1.1°C because of danger of breakage from ice formation. Glass is not apt to be stored at these temperatures, whereas plastic containers might.

Polyethylene bottles were filled with hot juice (reconstituted concentrate) from the pasteurizer, sealed with screw caps and cooled to about 15.6°C under chilled water sprays. Bottles were placed in cases of 6 and immediately stored at 1.1°C. After about 90 min, 13 bottles were selected at random from 22 cases and analyzed for initial AA. Twenty cases each (120 containers) were stored at −6.7, 1.1 and 10°C.

The 4-oz polystyrene bottles (glue-sealed foil closures) and 64 oz cartons (glue-sealed folded tops) contained fresh SSOJ, which had been pasteurized, cooled in successive heat exchangers, and filled cold under aseptic conditions. Three cases of 48 4-oz bottles and 20 cases of 6 64-oz cartons were taken from commercial production lines (from single blender tanks of juice) and a random sampling of containers analyzed at the plant. Remaining samples were stored at −6.7, 1.1 and 10°C.

Frozen concentrated orange juice (45° Brix)

Containers tested were the widely used 6-oz cans of paper laminated with polyethylene having aluminum ends; and 12-oz rectangular cartons with laminated sides of paper, aluminum foil and polyethylene and having heavy polyethylene film lining the inside of the paperboard ends. Two cases of 48 6-oz cans were taken from the freezing tunnel, in a citrus processing plant, transferred to wire baskets, placed in turbulent −20.5°C air overnight before determining initial AA values, then divided, and stored at −15, −6.7 and 1.1°C. One hundred 12-oz cartons of FCOJ were taken from a commercial freezing tunnel, transferred to wire baskets, exposed to turbulent air at −20.5°C overnight before determining initial AA values and storing at −15, −6.7 and 1.1°C.

This study of FCOJ was originally to be carried out over 12 months. However, studies on products at −6.7°C and 1.1°C had to be terminated earlier. Because these concentrates are not sterile, samples were subject to microbial deterioration at these temperatures, and were discarded when spoilage became apparent.

Reconstituted concentrates

Commercial orange concentrate was reconstituted to 13° Brix, the initial AA was determined and portions placed in open beakers, closed glass bottles, and open and closed plastic pitchers. All containers were 64 oz and were about three-quarters filled with juice. Samples

were stored at 4.4, 10 and 21.1°C. Periodic AA analyses were made to determine the rate of change under conditions possible in a home. Consumers would be expected to use reconstituted concentrates within a week, so these studies ran 7 days.

RESULTS & DISCUSSION

Initial AA levels

Initial levels of AA averaged about 50 mg % (mg AA/100 ml SSOJ) and ranged from about 48 to 52 (Table 1). These differences probably reflect the predominating cultivar in the product or season and climatic factors. Initial FCOJ level was 184 mg % and did not appear to reflect processes or to affect retention rates in different packaging materials. On these and storage samples, replicates generally did not vary by more than 0.5%.

Table 1—Initial AA levels for orange juice products in different containers

Products	Ascorbic acid mg %[a]
SSOJ	
Glass (7 oz)	48.6
Polyethylene (64 oz)	48.2
Polystyrene (4 oz)	51.5
Cardboard cartons (64 oz)	52.2
FCOJ	
Laminated paper/polyethylene with aluminum ends	184.4
Laminated paper/aluminum foil with heavy polyethylene lined ends	184.5

[a] All replicate sample analyses varied by 0.25% or less.

Single-strength orange juice

SSOJ packaged in glass retained about 90% of initial AA for over 4 months and 87% for 1 yr at 4.4°C (Fig. 1). A slight increase in AA was noted after about 200 days at the two lower temperatures. This probably indicated the onset of fermentation which may cause slight increases in AA. AA retention was progressively less at 10° and 15.6°C (84 and 79%, respectively). AA retention at 26.7°C was reduced to 67% after 8 months when assay was discontinued because the juice was unacceptable in flavor and appearance.

AA retention in 64-oz polyethylene bottles (Fig. 2) was considerably less than in glass. Samples stored at −6.7°C and 1.1°C retained 82 and 38.5%, respectively, after 6 months. The assay was discontinued at that time because at 1.1°C color and flavor were unacceptable, although at −6.7°C, control samples were satisfactory. Samples at 10°C retained 32% of their AA content after 3.5 months when the assay was discontinued because of spoilage.

AA retention in polystyrene bottles was significantly less (Fig. 3) than in polyethylene or glass. Wall thickness of the polystyrene was 1/2 that of the polyethylene and these containers had only 1/16th the capacity. The 4-oz polystyrene bottles are designed for rapid use, continuous refrigerated handling and quick turnover (2 wk or less). High AA protection would not normally be required. In these samples AA assay was discontinued after 3 wk at 10°C and 6 wk at −6.7°C and 1.1°C due to product deterioration. After 6 wk at −6.7°C AA retention was 80.5% and at 1.1°C, 21%. Juice stored at 10°C for 3 wk retained 37%. However, all samples retained about 90% or more for about 1 wk.

AA retention in 64-oz cardboard cartons was about the same as that in the small polystyrene bottles (Fig. 4). Although observations extended 1 to 2 wk longer in the cardboard cartons, data for corresponding temperatures and times could be compared. AA retention in cartons after 6 wk at −6.7°C was 81.5% while in polystyrene bottles it was 80.5%. For the same time period at 1.1°C the cartons retained 39% while the polystyrene retained 21%. On the other hand after 3 wk at 10°C, cartons retained 26.5% and polystyrene 37%. At −6.7°C and 1.1°C both container types retained about 80% or better for the normal expected 2-wk turnover time.

Thus, among the four types of containers commonly used for the distribution of chilled orange juice, crown-capped glass bottles resulted in better AA protection even at higher storage temperatures. Hermetically sealed polyethylene containers were next, while losses were greater in polystyrene bottles and waxed cartons. This may have been due to the thin walls and higher surface area as well as the package material.

Figure 5 indicates the relative stability at the three temperatures for FCOJ in rectangular fiber cartons with aluminum foil barriers on the inner surface while

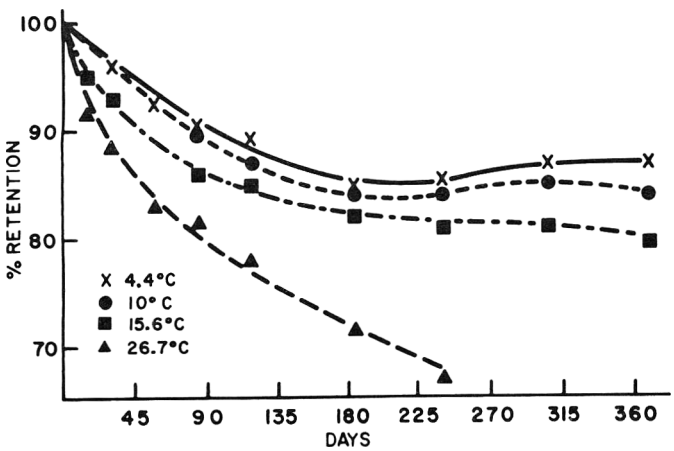

Fig. 1—AA retention in SSOJ in (crown capped, 7 oz) glass bottles.

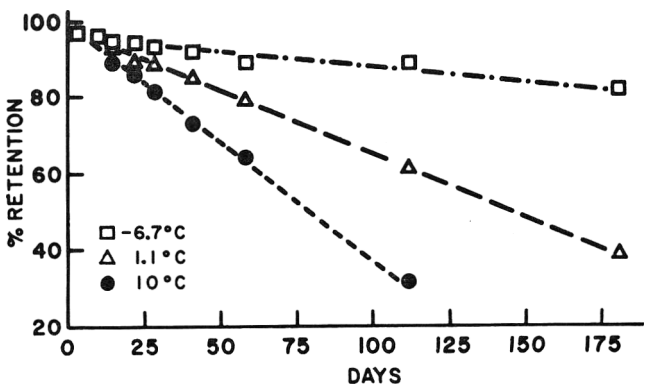

Fig. 2—AA retention in SSOJ in (64 oz) polyethylene bottles.

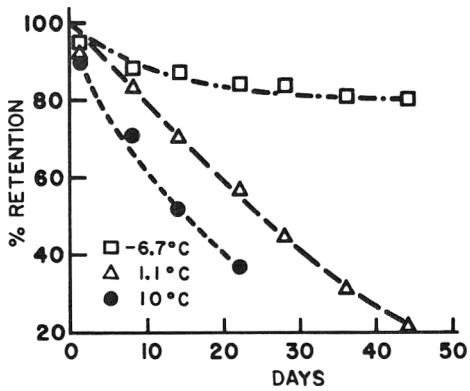

Fig. 3—AA retention in SSOJ in (4 oz) polystyrene containers.

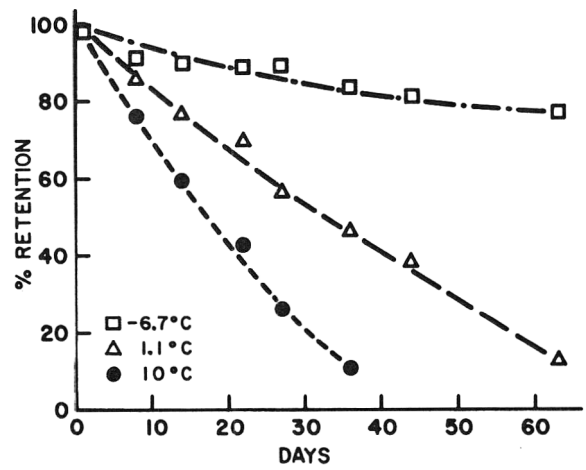

Fig. 4—AA retention in SSOJ in (1/2 gal) waxed cardboard cartons.

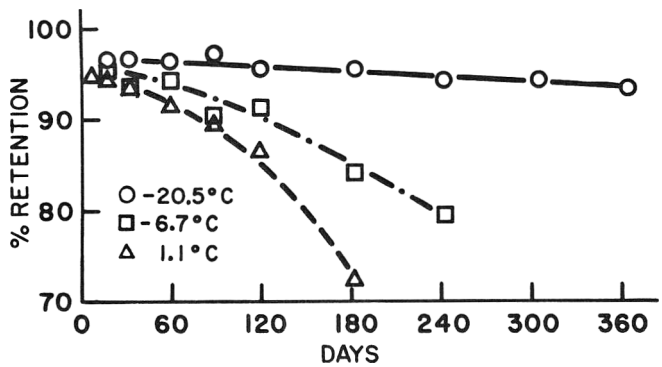

Fig. 5—AA retention in FCOJ in (12 oz) foil-lined fiberboard cartons.

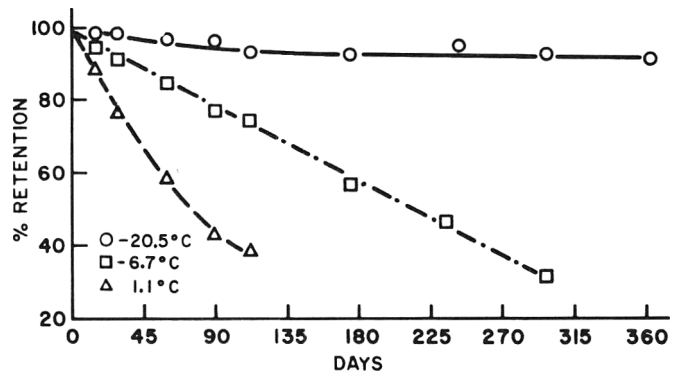

Fig. 6—AA retention in FCOJ in (6 oz) polyethylene lined fiberboard cans.

Figure 6 shows similar data for concentrates in a fiber can with polyethylene on the inner surface. There was little difference in AA retention at −20.5°C after 1 yr; fiber/polyethylene retained 91.5% and foil retained 93.5%. At higher storage temperatures however, the foil barrier resulted in superior retention. After 8 months at −6.7°C, FCOJ in foil-lined cartons retained 79% of the initial AA, while that in polyethylene lined cans retained 43%. Foil also proved superior at 1.1°C retaining 89% after 3 months, while the polyethylene retained 44%.

Simulated home use tests

After 7 days, samples of SSOJ reconstituted from concentrate retained 80–85% of their original AA content regardless of temperature (4.4°, 10° or 21.1°C). Differences between open and closed containers were about 1–2% at 4.4°C and 10°C and 3–5% at 21.1°C with the close containers, in each case, resulting in slightly better retention of AA. Retention differences between plastic and glass refrigerator containers were less than 1% in all cases. Samples at 21.1°C showed evidence of fermentation after 3 or 4 days. However, the data indicated that about 90% or more of the original AA would be retained during the first 4 days at 4.4°C or 10°C, during which time most consumers probably would use the reconstituted orange juice.

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A RAPID MICRO TECHNIQUE FOR TESTING BIODEGRADABILITY OF NYLONS AND RELATED POLYAMIDES

INTRODUCTION

ONE OF THE most popular methods for determining the biodegradability of a material is by Biochemical Oxygen Demand (BOD) (Anonymous, 1970), which may be performed manometrically or titrametrically. Mills and Stack (1955) have discussed the limitations of the BOD test when used for determining the biodegradability of organic compounds. Bunch and Chambers (1967) reported a static system using settled sewage as inoculum for determining the extent of degradation of organic compounds, while infrared, gas chromatography and thin-layer chromatography have been used to trace the evolution of products during degradation of hydrocarbons (Anonymous, 1970; Bartha and Atlas, 1971). Several techniques have been reported to check surfactant biodegradability (Setzkorn, 1964; Snow, 1965; Weaver and Coughlin, 1964) and the simplest of these is the River-Die-Away test (Renn et al., 1964; Sweeney and Foote, 1964; Weaver and Coughlin, 1964). The Activated Sludge test allows continuous feeding of the test sample and biodegradability is measured by the standard Methylene Blue test (Anonymous, 1970). The "Shake-Flask Inoculum" has been adopted by the Sub-Committee of Biodegradability Test Methods of the Soap and Detergent Association (Snow, 1965) as a presumptive test procedure for determining biodegradability of surfactants.

In the field of pesticide degradation, Stojanovic et al. (1972) have estimated the extent of biodegradation from the CO_2 evolved during incubation, and pesticide-microbial interactions were observed through plate counts made from the incubated samples. The incubation method of Bartholomew and Broadbent (1949) was used and the CO_2 evolved was measured respirometrically.

Studies on the biodegradability of common plastics, such as polyethylene, polystyrene and polyesters were performed by Potts et al. (1971) using a mixture of four species of fungi. Biodegradability was estimated by the density of growth on solid surfaces containing the test material as a sole source of carbon. Studies on the burial of certain plastics were also conducted and biodegradability measured by weight loss after a definite period of time.

Hynes and Pateman (1970) reported on the utilization of low molecular weight amides by *Aspergillus nidulans* as nitrogen sources. The study was conducted by adding amides as sole nitrogen sources to glucose-minimal medium and comparing growth with that in a "nitrogen-free" medium.

Hong and Barker (1972) described a Brevibacterium capable of degrading dl-erythro-3,5-diamino-hexanoate. They indicated that they were unable to isolate this organism from several garden soils and ultimately isolated it from sewage. A Brevibacterium of apparently identical characteristics and capable of degrading nylon salt (hexamethylene diamine adipate) was isolated by the present authors from a sandy loam.

Preliminary work on the biodegradability of polyamides (Moreno, 1973) indicated excellent agreement between mean microbial counts over 14 days and mean rate of production of CO_2/day under aerobic conditions for a range of test materials.

The purpose of this paper is to outline a method of determining the biodegradability of polymers, specifically polyamides, on a micro scale.

MATERIALS & METHODS

PRELIMINARY WORK with a number of mixed sources of microorganisms at different temperatures pointed to the value of using soil as a source, either as a slurry or as inoculum to an artificial medium. The latter was found to be superior in that degradation response was more rapid, and also the C:N ratio of this medium could be more easily controlled and maintained at a high level. Optimal incubation temperature was 35°C.

A sandy loam was used to develop the 20% soil slurries and 20 mg of test material was added to 20 ml of the slurries. 50-ml flasks, closed with resealable rubber stoppers, were used and the effect of aerobic and anaerobic conditions investigated by developing an initial 100%, 20% and 0% oxygen atmosphere.

Inoculation of the same soil to nutrient broth and incubating for 4 days provided the mixed soil micro-flora source for 1 ml inoculations of the artificial medium. 20 mg of test material were added to 20 ml of this medium, in 50-ml flasks, which contained 5g of a pancreatic digest of casein plus 1g dextrose per liter, and the flasks were closed with resealable rubber stoppers. After sterilization at 121°C for 15 min, the samples were inoculated with 1 ml of the soil micro-flora source and the headspace flushed with O_2 or He to provide aerobic or anaerobic conditions. Controls consisted of samples tested identically, but containing no test material.

The flasks from either procedure were then incubated at 35°C for up to 26 days and 1-ml headspace samples were withdrawn at regular intervals at first and less frequently later for CO_2 measurement using the Fisher-Hamilton Gas Partitioner. After each determination, the flasks were flushed with the appropriate gas (O_2 or Ar/He) to restore the initial headspace atmosphere. Figure 1 shows a schematic of the flasks currently in use.

RESULTS

Nylon 6,6 and nylon salt

Nylon 6,6 is a copolymer of hexamethylene diamine and adipic acid. The product is produced commercially from nylon salt, which is the acid and amine salt. Respirometric studies of headspace CO_2 with nylon 6,6 and nylon salt are summarized in Table 1.

The soil slurry microorganism source described previously was used. The extent

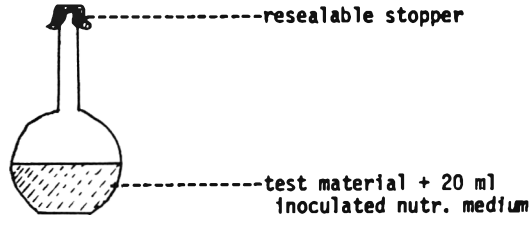


Fig. 1—50 ml flask with resealable stopper for use in biodegradation studies.

of degradation of the test materials was compared with casein and a control using the Student-Newman-Keule (SNK) procedure at the 1% confidence level for comparison among treatment means. It can be seen that under anaerobic conditions, nylon salt does not degrade at all but at 20% O_2 shows evidence after 2 days and after 1 day at 100% O_2.

Casein degrades under anaerobic conditions after 10 days but otherwise is generally not significantly different from nylon salt.

Nylon 6,6, however, shows no evidence of degradation under any atmospheric conditions.

Effect of irradiation

The effect of UV irradiation for 12 and 24 hr on a polymer of 2,4-pentane diamine and adipic acid under aerobic conditions was studied. Figure 2 shows how UV irradiation has no effect on respiration compared with a control and after 21 days there is no significant difference between treatments at the 5% level.

Low molecular weight models

The biodegradation of several low molecular weight nitrogen-containing compounds (including amides) were studied in order to assess the importance of functional end groups and molecular weight on biodegradability. Table 2 shows the structures and codes of the compounds used.

The one peptide case

The extent of degradation of a one-peptide model compound, hexamethylamine glutaramide (BJ4), is shown in Figure 3, where it is clear that no degradation occurs under anaerobic conditions, but that the compound readily breaks down in the aerobic situation. In the case of this compound it takes 4 days to show a significant difference from the control at the 5% level, indicated by the LSD, but 8 days to show significant difference from all treatments.

The dipeptide, an imide and blocking of the unipeptide

Figure 4 illustrates the effect of blocking the unipeptide, BJ4, with the $(CH_3)_3$-CO-CO- group at the NH- terminal (BJ7) and also the effect of blocking the the NH- terminal with this group and the -COOH terminal with the hydrazide ($-NHNH_2$) (BJ8). The respiratory activity in the presence and absence of these compounds is compared with the dipeptide (BJ5), based on BJ4, and also the imide, N-acetyl valeramide (BJ6). The dipeptide consists of two units of hexamethylene diamine and one unit of glutaric acid, so that the compound has an amide group at each end. Using the SNK test at the 1% level, it appears that the dipeptide significantly retards microbial respiration during the first day under aerobic conditions. It is not until after 4 days, however, that the imide shows significant respiratory response, compared with the control and all other treatments. This increased respiration due to the presence of the imide becomes even more pronounced and after 26 days the CO_2 level in flasks containing this material is considerably higher than any other treatment. There is evidence after 13 days, based on the SNK procedure, that one of the blocked compounds, BJ7, shows significant degradation. The dipeptide and the second blocked unipeptide (BJ8) do not give evidence of degradation.

Using the same procedure for comparing treatment means under anaerobic con-

Table 1—Mean cumulative % CO_2 (2 samples per mean)[a]

	Time (days)							
Initial Atm	1	2	4	7	10	14	21	Test material
0% O_2	.11	.37	.68	.96	1.32	1.64	2.32	Control
	.17	.42	.77	1.21	1.85	2.36	3.11	Nylon 6,6
	.55	1.18	1.83	2.90	3.88	4.88	6.24	Nylon salt
	.33	.88	5.08	9.49	12.83	16.15	18.96	Casein
20% O_2	.37	.75	1.42	2.14	2.09	3.61	4.57	Nylon 6,6
	.63	1.03	1.80	2.53	2.94	3.65	4.80	Control
	1.00	3.80	9.75	18.03	23.93	30.18	36.52	Nylon salt
	1.23	1.95	5.28	15.23	22.07	27.70	31.83	Casein
100% O_2	.63			2.54	3.10	3.77	4.68	Nylon 6,6
	.83			2.27	2.75	3.47	4.05	Control
	1.06			23.92	28.65	32.15	36.63	Casein
	2.37			28.61	34.57	38.67	42.46	Nylon salt

[a] Means joined by a straight line are not significant at the 1% level according to the SNK test; all other comparisons are significant within days and atmospheres.

Table 2—Structures and codes of compounds used in biodegradability study

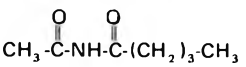
BJ4

NH_3^+-$(CH_2)_6$-NH-CO-$(CH_2)_3$-COO^-

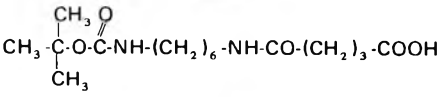
BJ5

NH_2-$(CH_2)_6$-NH-CO-$(CH_2)_3$-CO-NH-$(CH_2)_6$-NH_2

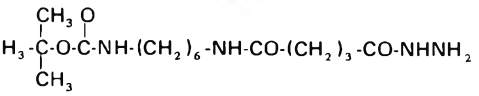
BH6



BJ7



BJ8



ε-ACA

NH_2-$(CH_2)_5$-COOH

Blocked ε-ACA

HCl · NH_2-$(CH_2)_5$-$COOCH_3$

ditions, none of these materials was biodegradable when compared with a control.

Blocked and nonblocked ϵ-aminocaproic acid (ϵ-ACA)

ϵ-ACA can be used as a constituent of alternating copolymers such as nylon 2/6 and alt. nylon 3/4/6 and, as is shown later, this type of polymer shows promise as a biodegradable packaging material. Table 3 shows how ϵ-ACA was highly biodegradable, but blocking of this compound inhibited degradation to the extent that the cumulative CO_2 after 35 days in the presence of the blocked compound was not significantly different from the control.

A brief look at the effect of blocking ϵ-ACA on respiration (Fig. 5) shows that this compound dramatically inhibited microbial growth during the first 2 days of incubation and thereafter the rate of respiration increased rapidly. For this reason it appears that a long incubation period is required to assess the biodegradability of this compound.

Figure 6 summarizes these results and it can be seen how the presence of a terminal acid group apparently favored bio-

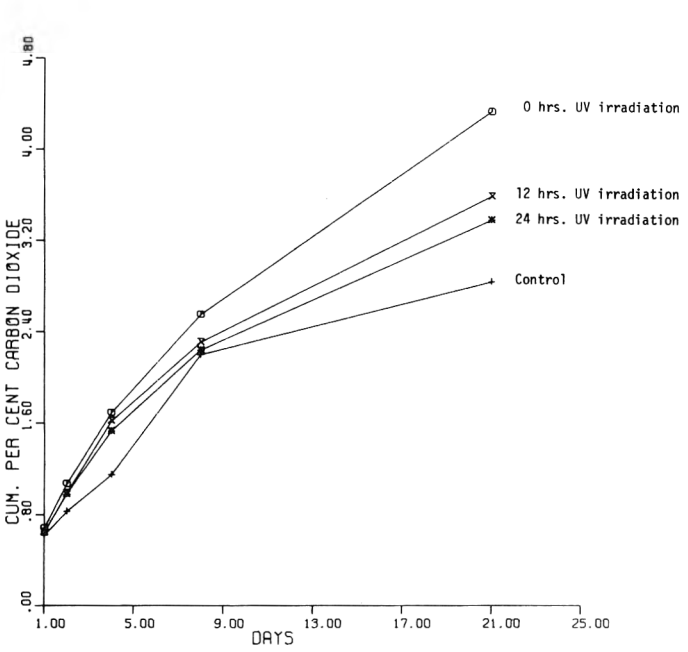


Fig. 2—Biodegradability of UV-irradiated samples of a polymer of 2,4-pentane diamine and adipic acid.

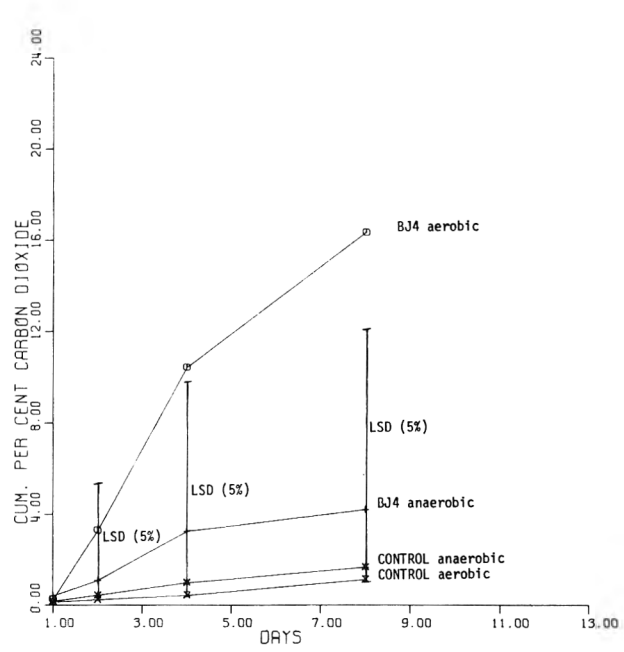


Fig. 3—Biodegradability of BJ4 under aerobic and anaerobic conditions.

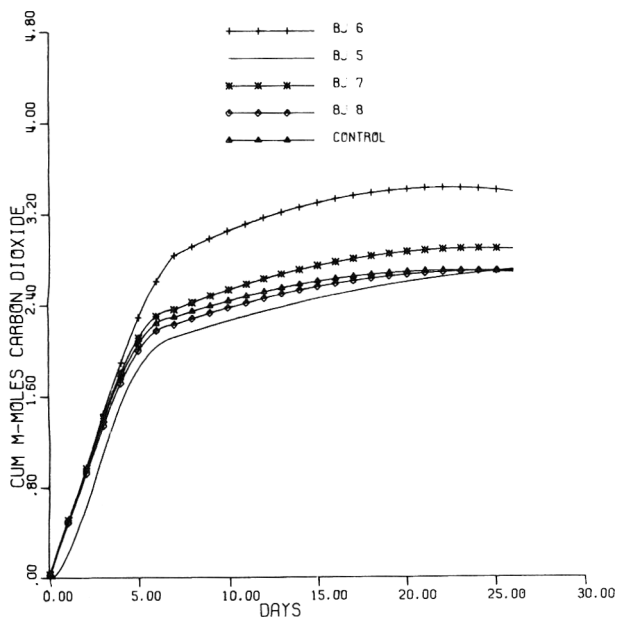


Fig. 4—Biodegradability of several low molecular weight models.

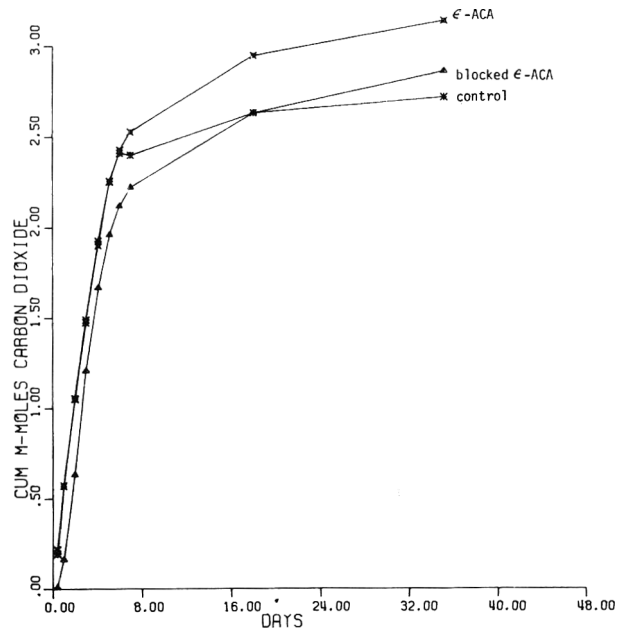


Fig. 5—Effect of blocking on the biodegradability of ϵ-ACA.

degradability. Because of the effect of the terminal functional group, the relationship between biodegradability and molecular weight was not clearly defined. For example, even though the molecular weights of BJ5 and BJ7 were almost identical (328 and 329), the former was not biodegradable but the latter was.

Alt. nylon 2/6 and alt. nylon 3/4/6

Figure 7 shows the cumulative mmoles of CO_2 accumulated in the presence of two polyamides, alt. nylon 2/6 (alt. n2/6) and alt. nylon 3/4/6 (alt n3/4/6) under aerobic conditions. Once again, using the SNK test at the 1% confidence level, alt. n2/6 showed significantly higher accumulation of CO_2 after 7 days compared to the control and accumulation up to 26 days served only to substantiate this difference. Alt. n3/4/6 does not degrade under aerobic conditions and an SNK test at the 1% level showed that neither degraded under anaerobic conditions.

Polycaprolactone, polyglycine, alt. nylon 6,6/nylon 2, alt. nylon 2/p-amino benzoic acid, nylon 6

Table 2 shows that only one of these polymers, polycaprolactone, degraded sufficiently to make a significant contribution to headspace cumulative CO_2 after 35 days of incubation. The biodegradability of polycaprolactone is consistent with the findings of others (Potts et al., 1971).

Table 3—Cumulative headspace CO_2 (% of control) after 35 days in the presence of certain low molecular weight models and several polymers[a]

Compound	Cum. CO_2 (% of control)
Alt. nylon 6,6/nylon 2	96.2
Polyglycine	97.0
Alt. nylon 2/p-aminobenzoic acid	99.4
Nylon 6	99.7
Control	100.0
Blocked ϵ-aminocaproic acid	105.1
Polycaprolactone	112.1
ϵ-aminocaproic acid	115.4

[a] Means joined by a straight line are not significantly different using the SNK test at the 5% level.

DISCUSSION & CONCLUSIONS

A PRACTICAL TEST for biodegradation should be rapid, accurate, reliable and simple. The foregoing method can be used to detect the biodegradability of small quantities of readily biodegradable test materials in as little as 1 day. Less readily biodegradable materials can be evaluated in 4—7 days but some polymers may take 1 month. By increasing the initial O_2% headspace concentration up to 100% we have shown that a significant reduction in the time required to demonstrate biodegradability can be achieved. In many cases the biodegradability of a test material was not detected under anaerobic conditions.

The inoculum used should contain as wide a range of microorganisms as possible and for this reason we used a soil source. By inoculating a standard medium (pancreatic digest of casein and dextrose) with soil microorganisms in the presence of test material, (replicated four times) the within cell error was low enough to detect respiration differences of ±5%, as compared with the control, 99% of the time. Considerably more replication variance was found where soil slurries were used as inoculum plus nutrient source in the presence of test material.

The method proposed offers considerable simplicity when compared with other methods for determining the biodegradability of organic compounds. In cases where the time-respiration profile is not of interest, larger flasks (250 ml) could be used and a one-time analysis per-

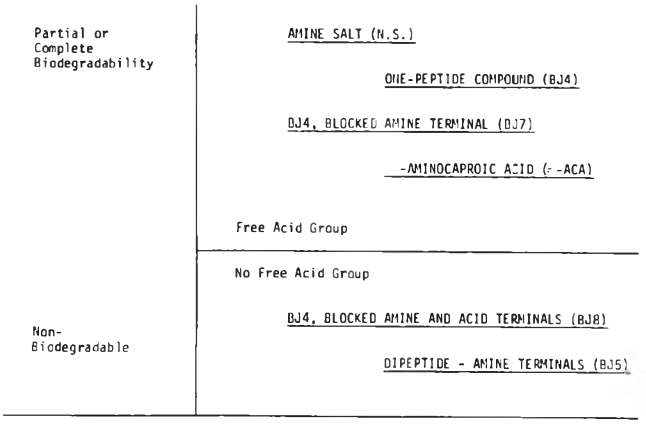


Fig. 6—Summary of results showing presence of acid terminal group favoring biodegradation.

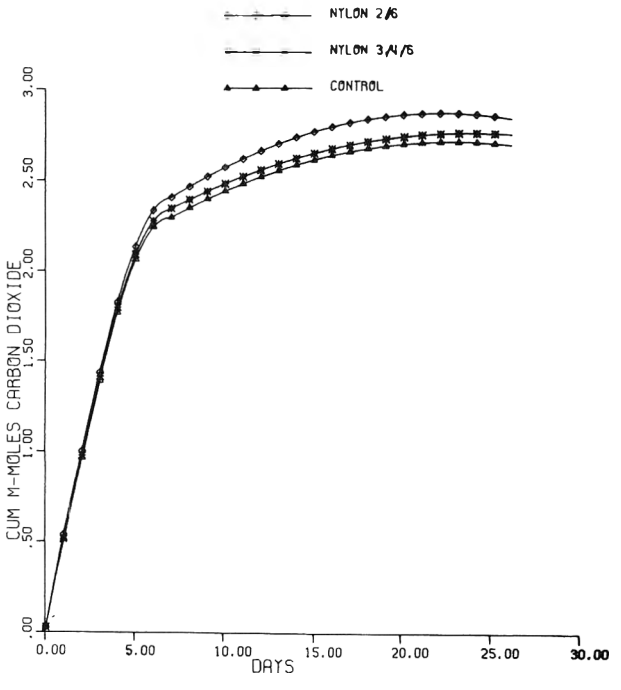


Fig. 7—Biodegradability of alternating copolymers alt. nylon 2/6 and alt. nylon 3/4/6.

formed after a specific period of time. Using the Fisher-Hamilton Partitioner, this analysis takes 4–5 min per sample. Samples smaller than 20 mg could be used but a longer incubation period would be required to detect biodegradability.

From studies with low molecular weight models, we have shown that the presence of a terminal acid group favors biodegradability over the range of compounds used.

Several N-containing polymers such as nylon 6,6, alt. nylon 3/4/6, polyglycine, alt. nylon 6.6/nylon 2, nylon 6 and alt. nylon 2/p-amino-benzoic acid were not biodegradable. However, alt. nylon 2/ nylon 6 showed significant cumulative CO_2 after 7 days when compared with the control, and polycaprolactone was readily biodegradable. Although much work needs to be done on the physical properties of alt. nylon 2/nylon 6 as a packaging material, the thermal stability and melting point (268–270°C) of the polymer point to its potential use for this purpose.

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FORMULAE FOR PREDICTING GAS EXCHANGE OF FRESH PRODUCE IN POLYMERIC FILM PACKAGE

INTRODUCTION

CONTROLLED ATMOSPHERIC storage has been successfully utilized for extending shelf life of fresh produce (Anonymous, 1967; Dewey et al., 1969). For this storage, the gaseous compositions of atmosphere around fresh produce are artificially modified to regulate its respiration rate. This is accomplished through the addition of CO_2 and/or other gases to the surrounding atmosphere.

Several workers investigated economical procedures for creating favorable gaseous compositions in microatmosphere around fresh produce through polymeric film packaging (Daun et al., 1973; Gilbert et al., 1971; Hansen, 1963; Hardenburg, 1963, 1971; Karel and Go, 1964; Nichols and Hammond, 1973). Daun and Gilbert (1974) presented extensive reviews on published investigations on biochemical and physical characteristics of these packages. Several workers (Jurin and Karel, 1963; Tolle, 1962, 1971; and Veeraju and Karel, 1966) developed mathematical procedures for optimizing the packaging parameters of post climacteric fresh produce. These workers assumed a constant respiration quotient of fresh produce. Henig and Gilbert (1972) improved their procedures by mathematically simulating O_2 and CO_2 gas exchange of the package. For this simulation, they assumed variable respiration quotients. The model consists of two simultaneous first order ordinary differential equations together with boundary and initial conditions. They solved the mathematical models through the use of a simple finite difference procedure. They investigated the influence of several packaging parameters on transient and equilibrium state gaseous compositions in sample packages. Numerical solutions are applicable to the specific values of packaging parameters, which are used for deriving these solutions. There are no packaging parameters explicitly included in the solutions. Therefore, the parameters are optimized through a trial and error technique when they are utilized.

In the present investigation, a new mathematical model is developed for simulating the gas exchange of a fresh produce package. For this development, Henig and Gilbert's model is slightly modified through the careful examination of available respiration rate data. Analytical solutions are then obtained for this model. From these solutions, simple algebraic formulae are derived for the optimization of packaging parameters.

DERIVATION OF FORMULAE

DATA on the post climacteric respiration rates of fresh produce (Henig, 1972; Veeraju and Karel, 1966) are carefully examined and we observe the following relationships. The rates of both oxygen consumption and carbon dioxide evolution by fresh produce are likely affected by atmospheric carbon dioxide and oxygen concentrations. Therefore, changes in respiration rates are approximated with the combination of the following linear equations.

Rate of O_2 consumption

$$R_{yi} = o_i y + P_i z + q_i \tag{1}$$

Rate of CO_2 evolution

$$R_{zi} = d_i y + e_i z + f_i \tag{2}$$

for $y_{i+1} \leq y \leq y_i$; $z_i \leq z \leq z_{i+1}$; $(i = 0,1,2,\ldots,n)$.

In published investigations, formulae were derived by assuming that the rate of oxygen consumption was affected by oxygen concentration in surrounding atmosphere and also that the rate of carbon dioxide evolution was affected by the carbon dioxide concentration in the atmosphere. Therefore, Eq. 1 and 2 are more generally applicable when they are compared with these formulae.

The mass balance of gas components in a fresh produce package is represented by the following ordinary differential equations.

$$\frac{dy}{d\tau} = \frac{Sk_y}{V_f}(y_a - y) - \frac{W}{V} R_{yi} \tag{3}$$

$$\frac{dz}{d\tau} = \frac{Sk_z}{V_f}(z_a - z) + \frac{W}{V} R_{zi} \tag{4}$$

for $y_i \leq y \leq y_{i+1}$; $z_{i+1} \leq z \leq z_i$; $(i = 0,1,2,\ldots,n)$ (5)

$$\tau_i = t - t_i \tag{6}$$

$$y = y_{oi} \text{ and } z = z_{oi} \text{ at } \tau = 0 \tag{7}$$

The above equations are solved analytically by applying Laplace transformation. The solutions obtained are given below:

Transient state O_2 concentration

$$y = y_{eqi} + \frac{y_{oi} r_1^2 + N_1 r_1 + N_2}{r_1(r_1 - r_2)} \cdot \exp(r_1 \tau) + \frac{y_{oi} r_2^2 + N_1 r_2 + N_2}{r_2(r_2 - r_1)} \cdot \exp(r_2 \tau) \quad y_i \leq y \leq y_{i+1} \tag{8}$$

where

$$N_1 = y_{oi}\left(\frac{S}{V_f} k_z - \frac{W}{V} e_i\right) + \frac{S}{V_f} \cdot k_y y_a - \frac{W}{V} q_i - \frac{W}{V} P_i z_{oi} \tag{8.1}$$

$$N_2 = \left(\frac{S}{V_f} k_y y_a - \frac{W}{V} q_i\right)\left(\frac{S}{V_f} k_z - \frac{W}{V} e_i\right) - \frac{W}{V} p_i\left(\frac{S}{V_f} k_z z_a + \frac{W}{V} f_i\right) \tag{8.2}$$

$$y_{eqi} = \frac{N_2}{r_1 r_2} \tag{8.3}$$

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The symbols r_1 and r_2 represent the roots of the following quadratic equation:

$$r^2 + \left\{ (k_y + k_z)\frac{S}{V_\iota} + (o_i - e_i)\frac{W}{V} \right\} r + \left(\frac{S}{V_\iota} k_y + \frac{W}{V} o_i\right)\left(\frac{S}{V_\iota} k_z - \frac{W}{V} e_i\right) + \left(\frac{W}{V}\right)^2 p_i d_i = 0 \qquad (8.3)$$

Transient state CO_2 concentration

$$z = z_{eqi} + \frac{z_{oi} r_1^2 + M_1 r_1 + M_2}{r_1(r_1 - r_2)} \cdot \exp(r_1 \tau) + \frac{z_{oi} r_2^2 + M_1 r_2 + M_2}{r_2(r_2 - r_1)} \cdot \exp(r_2 \tau) \qquad z_{i+1} \geqslant z \geqslant z_i \qquad (9)$$

where

$$M_1 = z_{oi}\left(\frac{S}{V_\iota} k_y + \frac{W}{V} o_i\right) + \frac{S}{V_\iota} k_z z_a + \frac{W}{V} f_i + \frac{W}{V} d_i y_{oi} \qquad (9.1)$$

$$M_2 = \left(\frac{S}{V_\iota} k_y + \frac{W}{V} o_i\right)\left(\frac{S}{V_\iota} k_z z_a + \frac{W}{V} f_i\right) + \frac{W}{V} d_i \left(\frac{S}{V_\iota} k_y y_a - \frac{W}{V} q_i\right) \qquad (9.2)$$

$$Z_{eqi} = \frac{M_2}{r_1 r_2} \qquad (9.3)$$

It is possible that Eq. 8.3 has double roots instead of two single roots, although this is an extremely rare situation. Formulae applicable to this situation are not presented because of space limitations.

The analytical solutions presented above may be used for estimating transient and equilibrium state gas compositions in most fresh produce packages because of fairly general assumptions imposed. However, the use of these solutions requires somewhat complicated computations. Available data on post climacteric respiration rates of fresh produce show that the rate of O_2 consumption is not greatly influenced by CO_2 concentration in the surrounding atmosphere and that the rate of CO_2 evolution is not significantly affected by O_2 concentration. Therefore, we assumed that $p_i = d_i = 0$ in order to simplify the solutions derived although this simplification results in less generally applicable formulae. We obtained the following equations (10 and 11) by entering these assumed values into Eq. 8 and 9:

$$\frac{y_{eqi} - y}{y_{eqi} - y_{oi}} = \exp\left\{ -\frac{1}{V}\left(Sk_y/\iota + Wo_i\right)\tau \right\} \qquad y_i \geqslant y \geqslant y_{i+1} \qquad (10)$$

$$y_{eqi} = (y_a k_y/\iota - q_i W/S)/(k_y/\iota + o_i W/S) \qquad (10.1)$$

$$\frac{z_{eqi} - z}{z_{eqi} - z_{oi}} = \exp\left\{ -\frac{1}{V}\left(Sk_z/\iota - We_i\right)\tau \right\} \qquad z_{i+1} \geqslant z \geqslant z_i \qquad (11)$$

$$z_{eqi} = (z_a k_z/\iota + f_i W/S)/(k_z/\iota - e_i W/S) \qquad (11.1)$$

Time after packaging, which is required for a fresh produce package to reach equilibrium state gas exchange, may be easily estimated from Eq. 10 and 11. The gas exchange of a fresh produce package practically reaches an equilibrium state when transient state gas concentration differs from equilibrium state concentration by 0.001 or 0.1%. Therefore, we have the following formulae:

$$t_{eqy} = \sum_{i=0}^{n-1} \frac{V}{Sk_y/\iota + Wo_i} \cdot \ln\left(\frac{y_{eqi} - y_{oi}}{y_{eqi} - y_{i+1}}\right) + \frac{V}{Sk_y/\iota + Wo_n} \cdot \ln\left(10^3 \cdot |y_{eqn} - y_{on}|\right) \qquad (12)$$

$$t_{eqz} = \sum_{i=0}^{n-1} \frac{V}{Sk_z/\iota - We_i} \cdot \ln\left(\frac{z_{eqi} - z_{oi}}{z_{eqi} - z_{i+1}}\right) + \frac{V}{Sk_z/\iota - We_n} \cdot \ln\left(10^3 \cdot |z_{eqn} - z_{on}|\right) \qquad (13)$$

Eq. 10.1 and 11.1 may be used for the optimization of packaging parameters. From these equations, we may estimate the proper gas permeability of polymeric film and the proper weight of fresh produce in one package. For example, the permeability of film, which produces desired equilibrium gaseous composition, is determined by the following equations:

$$k_y/\iota = \left\{ (y_{eqi} o_i + q_i) W/S \right\} / (y_a - y_{eqi}) \qquad (14)$$

$$k_z/\iota = \left\{ (z_{eqi} e_i + f_i) W/S \right\} / (z_{eqi} - z_a) \qquad (15)$$

In some cases, fresh produce is packed with polymeric films of two or more different types. Formulae applicable to these packages may be easily obtained from the above equations through the following substitutions.

$$Sk_y/\iota = S\sum_{\iota=1}^{N} a_\iota k_{y\iota}/\iota$$
$$Sk_z/\iota = S\sum_{\iota=1}^{N} a_\iota k_{z\iota}/\iota \qquad (16)$$

SAMPLE CALCULATION

EQUATIONS 10 and 11 may be used for estimating transient state gas concentrations in a fresh produce package. Sample calculations for this estimation are presented below.

The respiration rate constants of field tomatoes are determined at 21°C in CO_2 free atmosphere. These constants are:

$$R_{y1} = q_1 = 23.135 \qquad 0.21 \geqslant y \geqslant 0.1153 \qquad (17.1)$$

$$R_{y2} = o_2\, y = 200.65y \qquad 0.1153 \geqslant y \geqslant 0.040 \qquad (17.2)$$

$$R_{z1} = f_1 = 18.525 \qquad 0 \leqslant z \leqslant 0.092 \qquad (17.3)$$

$$R_{z2} = f_2 = 12.187 \qquad 0.092 \leqslant z \qquad (17.4)$$

The same tomatoes are placed in an RMF-61 film package. The parametric values of this package are:

$$k_y/\iota = 0.875 \text{ (cc of } O_2 \text{ / (hr in.}^2 \text{ atm))}$$

$$k_z/\iota = 4.505 \text{ (cc of } CO_2 \text{ / (hr in.}^2 \text{ atm))}$$

$$S = 53 \text{ in.}^3$$
$$V = 843 \text{ cc}$$
$$W = 0.472 \text{ kg}$$

Eq. 10 and 10.1 are used to estimate oxygen concentrations in the package. In order to calculate the concentrations within the range of y in which R_{y1} is applicable, a dummy equilibrium state concentration should be estimated by using Eq. 10.1.

$$y_{eqi} = \frac{0.21 \times 0.875 - 23.135 \times 0.472/53}{0.875 + 0} \doteq -0.02547$$

Transient state y values are calculated by entering this y_{eq1} value into Eq. 10.

$$y = -0.02547 + (0.21 + 0.02547) \times \exp\left\{-\frac{1}{843}(53 \times 0.875)\tau_1\right\}$$

$$= -0.02547 + 0.23547 \times \exp\left\{-0.05501 \times \tau_1\right\} \quad (18.1)$$

The above equation is used until y becomes equal to 0.1153, which is the lower limit of y, to which R_{y1} is applicable. By entering y = 0.1153 to the above equation, we find that t = 9.35 hr. Beyond this time value, we should use another equation, which is obtained by entering an o_2 value into Eq. 10. An equilibrium state y value used in this equation is estimated as follows:

$$y_{eq2} = \frac{0.21 \times 0.875}{0.875 + 200.65 \times 0.472/53} \triangleq 0.069$$

When the above equilibrium state value is entered into Eq. 10, we have the following formula:

$$y = 0.069 + (0.1153 - 0.069) \times \exp\left\{-\frac{1}{843}(53 \times 0.875 + 0.472 \times 200.65)\tau_2\right\}$$

$$= 0.069 + 0.0463 \times \exp\left\{-0.1674\,(t - 9.35)\right\} \quad (18.2)$$

$$t \geqslant 9.35$$

Transient state oxygen concentrations estimated by using Eq. 18.1 and 18.2 are shown in Figure 1 together with experimentally determined concentrations. Carbon dioxide concentrations are estimated through similar calculations by using Eq. 11 and 11.1. These concentrations are also shown in Figure 1. There is fair agreement between mathematically estimated and experimentally determined concentrations. Figure 2 shows results obtained for a VF-71 film package of fresh California tomatoes held at 15°C. There is fair agreement between mathematical and experimental concentrations.

DISCUSSION

AVAILABLE DATA on the gas exchange of fresh produce packages are utilized to examine the reliability of several formulae derived in the present investigations. The two junior authors of the present paper (Henig and Gilbert, 1972) collected extensive data on respiration characteristics of fresh tomatoes and bananas. From these data, they obtained empirical formulae for representing respiration rates of the sample produce. For deriving these formulae, they assumed that there was negligible influence of environmental CO_2 concentration on the rate of O_2 consumption and also negligible influence of environmental O_2 concentration on the rate of CO_2 evolution. Therefore, Eq. 10 through 11.1 are applicable to their respiration data. Equilibrium state O_2 concentrations, y_{eq}, and CO_2 concentrations, z_{eq}, in sample packages are estimated by using these equations (Table 1). Two different rates for the O_2 consumption of fresh produce are used for estimating y_{eq} values. One rate was determined in CO_2-free atmosphere. Another rate was determined in CO_2-accumulative atmosphere. It is interesting to note that the use of O_2 consumption rates determined in CO_2-free atmosphere results in the more accurate estimation of y_{eq} values when it is compared with the use of those rates determined in CO_2 accumulation atmosphere. Estimated y_{eq} and z_{eq} values agree with respective values determined experimentally.

Equilibrium time values, t_{yeq} and t_{zeq}, are calculated by using Eq. 12 and 13 (Table 1). It is observed from the table that there is close agreement between calculated and observed t_{yeq} values. The use of respiration rates, which were determined in CO_2-free atmosphere, results in more accurate estimation of t_{yeq} values when they are compared with the use of these rates, which were determined in CO_2 accumulative atmosphere. There are great differences between estimated and observed t_{zeq} values. These differences are likely caused by assumptions imposed for experimentally evaluating respiration rates.

It is observed from published data on transient state CO_2 concentrations (Henig and Gilbert, 1972; Jurin and Karel, 1963; Nichols and Hammond, 1973) that CO_2 concentration reaches a maximum level before asymptotically approaching to an equilibrium value as shown in Figures 1 and 2.

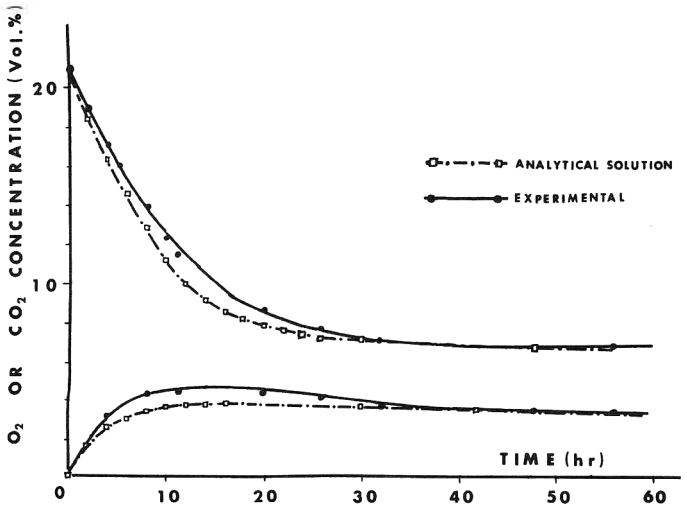

Fig. 1–Transient oxygen and carbon dioxide concentrations in an RMF-61 film package of field tomatoes at 21° C.

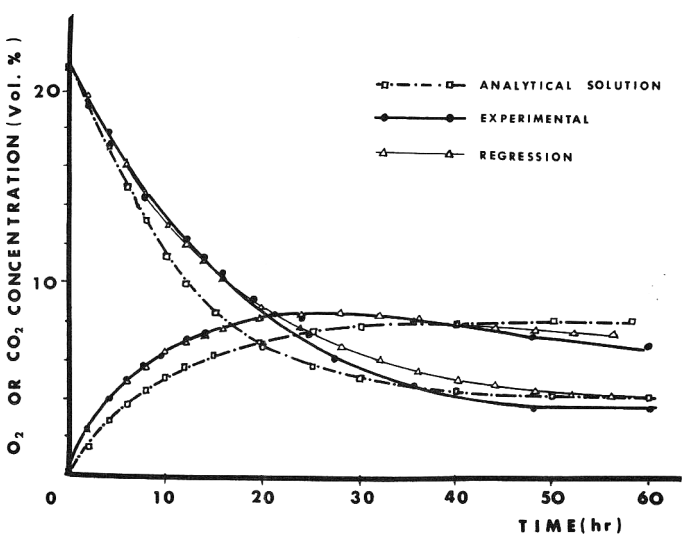

Fig. 2–Transient oxygen and carbon dioxide concentrations in a VF-71 film package of California tomatoes at 15° C.

Eq. 11 may be used for estimating transient state CO_2 concentrations if the rate of CO_2 evolution is not influenced with O_2 concentration in the surrounding atmosphere as stated previously. The simple analysis of this equation reveals that there is no maximum point in a transient state CO_2 concentration curve which is estimated by the equation. Eq. 9 is obtained by assuming that the rate of CO_2 evolution is affected with both O_2 and CO_2 concentrations in the surrounding atmosphere. When this equation is mathematically analyzed, it is observed that there could be a maximum point in a CO_2 concentration curve if the signs of the two terms, by which the two exponential terms of Eq. 9 are multiplied, are different from each other. This observation is confirmed through the nonlinear regression analysis of data on the transient state O_2 and CO_2 concentrations in fresh tomato and banana packages. For this analysis, the following equation is fitted to experimental data:

$$\xi = c_0 + c_1 e^{r_1 t} + c_2 e^{r_2 t} \quad (19)$$

It should be noted that the above equation is mathematically equivalent to Eq. 8 or 9. Parametric constants of Eq. 19, which are determined through the regression analysis, are shown in Table 2. Typical regression curves obtained through the analyses are shown in Figure 2. The results obtained clearly indicate that the O_2 consumption or CO_2 production rate of sample produce is affected with both O_2 and CO_2 concentrations in surrounding atmosphere. When Eq. 8 is com-

Table 1–Equilibrium concentrations and equilibrium time values of fresh tomato or banana packages

Fresh produce[a] and temp	Film	y_{eq} (%) Estimated CO_2 Ad.[b]	y_{eq} (%) Estimated CO_2 Ac.[c]	y_{eq} (%) Obs	z_{eq} (%) Est	z_{eq} (%) Obs	t_{yeq} (hr) Estimated CO_2 Ad.[b]	t_{yeq} (hr) Estimated CO_2 Ac.[c]	t_{yeq} (hr) Obs	t_{zeq} (hr) Est	t_{zeq} (hr) Obs
Greenhouse tomato Group II, 22.8°C	RMF-61	6.56	7.36	6.50	4.35	2.50	31.5	33.9	28.0	13.6	50.0
Field tomato Group II, 21°C	RMF-61	6.90	7.37	6.90	3.69	3.40	32.3	33.9	30.0	65.4	45.0
	VF-71	2.94	3.20	3.15	9.12	7.00[e]	42.0	45.2	40.0	49.8	75.0[e]
Field tomato Group III, 15°C	RMF-61	7.98	8.95	6.40	2.73	2.50[e]	32.8	35.0	30.0	11.4	66.0[e]
	VF-71	4.24	4.15	4.20	8.13	7.00[e]	48.7	56.9	48.0	35.8	70.0[e]
Calif. tomato Group II, 20°C	RMF-61	9.23	9.97	9.25	2.47	2.20[e]	40.2	41.7	38.0	11.0	50.0[e]
	VF-71	4.34	4.85	4.40	7.37	5.00[e]	49.4	56.0	48.0	44.6	75.0[e]
Banana, 15°C	RMF-61	0[d]	0[d]	1.66	–	–	20.7	20.7	22.0	–	–
Banana, 22.8°C	RMF-61	0[d]	0[d]	1.84	–	–	10.9	10.9	12.0	–	–

[a] The relative humidities of microatmosphere inside packages were more than 90%.
[b] Values listed were estimated by using the rates of O_2 consumption, which were determined in CO_2 free atmosphere. The atmosphere was created by adsorbing CO_2 gas with 15% KOH aqueous solution.
[c] Values listed were estimated by using the rates of O_2 consumption, which were determined in atmosphere, into which CO_2 gas generated by fresh produce was accumulated.
[d] Negative values were obtained.
[e] These values were estimated from transient state data since no equilibrium state CO_2 gas exchange was observed when experiments were terminated. However, the estimated values will be fairly close to actual values because the rates of changes in CO_2 concentrations were relatively small at the end of the experiments.

Table 2–Parametric constants of transient state gas concentration curves

Fresh produce and temp	Film	O_2 or CO_2	C_0	C_1	r_1	C_2	r_2
Greenhouse tomato Group II, 22.8°C	RMF-61	O_2	0.0665	0.080293	−0.10185	0.060298	−0.10178
		CO_2	0.0250	−0.22332	−0.10503	0.20044	−0.071096
Field tomato Group II, 21°C	VF-71	O_2	0.0315	0.10179	−0.099525	0.081818	−0.099518
		CO_2	0.0700	−0.43199	−0.074590	0.36113	−0.050753
Field tomato Group II, 15°C	VF-71	O_2	0.0371	0.10175	−0.064592	0.079091	−0.064504
		CO_2	0.0700	−0.25732	−0.082257	0.19638	−0.057159
Calif. tomato Group II, 20°C	RMF-61	O_2	0.0914	0.069455	−0.097253	0.049455	−0.097155
		CO_2	0.0248	−0.21043	−0.13116	0.18645	−0.097851
Calif. tomato Group II, 20°C	VH-71	O_2	0.0536	0.095795	−0.073425	0.065228	−0.073427
		CO_2	0.0500	−0.12989	−0.089417	0.082022	−0.038626
Banana, 15°C	RMF-61	O_2	0.0166	0.11322	−0.13414	0.092148	−0.13485
		CO_2	0.0704	−0.28553	−0.12033	0.21336	−0.10200
Banana, 22.8°C	RMF	O_2	0.0184	0.11272	−0.20273	0.093119	−0.20282
		CO_2	0.0430	−0.81963	−0.13533	0.76841	−0.10881

pared with Eq. 9, we observe that two constants, r_1 and r_2, in the former equation are identical to those in the latter equation. Table 2 shows that there are some differences in these constants of two regression equations, which are equivalent to Eq. 8 and 9, although differences are relatively small in most cases. As stated previously, Eq. 8 and 9 should be applied to the portion of transient state gas concentration curves, which correspond to one pair of line segments for approximating R_y and R_z curves. In the above regression analyses, Eq. 8 and 9 are applied to the entire portion of a gas concentration curve. Therefore, the observed differences in r_1 and r_2 values imply that more than one line segment are required for approximating an R_y or R_z curve.

The analytical formulae obtained in the present investigation clearly show the interrelationship of these parameters on the gas exchange of fresh produce. Therefore, these formulae will be greatly useful for the optimization of packaging parameters.

CONCLUSION

ANALYTICAL FORMULAE are obtained for estimating transient and steady state gas concentrations in a fresh produce package. These formulae are derived from ordinary differential equations by assuming that the rate of O_2 consumption or the rate of CO_2 production is influenced by O_2 and CO_2 concentrations in surrounding atmosphere. The formulae obtained are used for estimating the equilibrium state O_2 and CO_2 concentrations in fresh tomato or banana packages. There is fair agreement between concentrations estimated by the formulae and those determined experimentally. The analytical formulae are also utilized for estimating equilibrium time values for the gas exchange of the sample packages. There is fair agreement between mathematical and experimental time values for O_2 exchange. However, there are great differences between mathematical and experimental time values for CO_2 exchange. These great differences are likely caused by assumptions imposed for experimentally determining the respiration rate constants of fresh produce. The analytical formulae will be greatly useful for the optimization of packaging parameters since these parameters are explicitly included in the formulae.

NOMENCLATURE

a_l	$= S_l/S$. Ratio of surface area covered by film of one type and total surface area of a package through which gas is permeated.
c_0, c_1, c_2	Experimental constants used in Eq. 19. The symbol c_0 represents an equilibrium O_2 of CO_2 concentration.
d, e, f	Constants used in an equation for approximating a curve for the CO_2 evolution rate of fresh produce. (See Eq. 2.)
i	Dummy integer.
k	Permeability of polymeric film [cc of gas/(hr in.^{2} (atm/mil))].
ι	Thickness of polymeric film (mil).
M_1, M_2	Expressions defined by Eq. 9.1 and 9.2, respectively.
N	Number of different types of films, which are used in one fresh produce package. It is assumed that there are CO_2 and O_2 permeation through all of these films.
N_1, N_2	Expressions defined by Eq. 8.1 and 8.2, respectively.
n	Number of pairs of line segments, which are used for approximating a pair of curves obtained by plotting the rates of O_2 consumptions and of CO_2 evolution by fresh produce (See Eq. 1 and 2.)
o, p, q	Constants used in an equation for approximating a curve for the O_2 consumption rate of fresh produce. (See Eq. 1.)
R	Rate of respiration [cc of O_2 or CO_2/(hr Kg)].
r	Independent variable of Eq. 8.3.
r_d	Double roots of Eq. 8.3.
r_1, r_2	Two roots of Eq. 8.3.
S	Surface area of fresh produce package, through which O_2 and CO_2 gases are permeated. When film of more than one different type is used for a single package, this symbol represents total surface area, through which gas is permeated (in^2).
t	Time after packaging (hr).
V	Inside free volume of a package (cc).
W	Weight of fresh produce per one package (Kg).
y	Volumetric concentration of O_2 gas in a fresh produce package: The numerical value of y is identical to the partial pressure of O_2 in a package, which is expressed with the units of atmosphere (dimensionless).
z	Volumetric concentration of CO_2 gas in a fresh produce package. The numerical value of z is identical to the partial pressure of CO_2 in a package which is expressed with the units of atmosphere (dimensionless).
τ_i	$= t - t_i$. This represents time after y becomes equal to y_i or after z becomes equal to z_i. The symbol y_i is defined as the limit of (i − 1)th line segment for approximating a curve of R_y values. The symbol z_i is also defined in a similar way. (See Eq. 1 and 2.)
ξ	Dependent variable used in Eq. 19. This represents transient state O_2 and CO_2 concentrations.

Subscripts

a	Quantity applicable to atmosphere surrounding a package. For example, y_a represents oxygen concentration of atmosphere outside of a package.
eq	Quantity at actual equilibrium state.
eqi	Quantity at hypothetical equilibrium state which is applicable to the (i + 1)th line segment of a respiration rate curve. For example, y_{eqi} represents an O_2 concentration at the hypothetical equilibrium state, which may be estimated by Eq. 8.3, 10.1 or 11.1. This concentration is obtained by assuming the respiration rate equations 1 and 2 are applicable to infinitely large t values.
eqy, eqz	Respectively represent quantity applicable to equilibrium state O_2 and CO_2 gas exchanges.
i	When this subscript is used with o, p, q, d, e or f, it represents parametric constants applicable to the (i + 1)th line segment of a respiration rate curve. When this subscript is used with other symbols, it represents the quantity corresponding to a limit of a line segment of a respiration rate curve. In case of a quantity, which is related to a CO_2 evolution rate curve, the subscript represents a quantity related to the lower limit of a line segment of the latter curve.
i + 1	Quantity applicable to a limit of a line segment at a respiration rate curve. In case of a quantity which is related to an O_2 consumption rate curve, the subscript represents a quantity related to the lower limit of a line segment of this curve. In case of a quantity, which is related to a CO_2 evolution curve, the subscript represents a quantity related to the upper limit of a line segment of the latter curve.
ι	Dummy index, which represents each of the different types of films used in one package.
oi	Quantity applicable when $\tau = 0$.
y, z	Quantities which are related to O_2 and CO_2.
1, 2	First and second quantities, respectively.

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IMPROVEMENT OF SHELF LIFE OF PARTIALLY DEFATTED PEANUTS BY INTROMISSION OF NITROGEN INTO THE INTERSTICES OF THE PEANUTS

INTRODUCTION

ROASTED PEANUTS—one of the most popular snacks in the country—are high in calories. For the diet-conscious public a new product was developed to retain the peanut's original flavor, aroma and shape and to reduce its oil content and thus its calories (Pominski et al., 1969, 1970, 1971; Spadaro, 1969; Vix et al., 1966, 1967). This product called "partially defatted peanuts" has already been marketed in this country. Whereas full-fat peanuts contain about 50% oil, which accounts for 70% of their calories, the new peanut product has 50–60% of the oil removed.

To produce partially defatted peanuts, blanched peanuts (peanuts with skins removed) with an optimum moisture content of 5% are pressed at room temperature to remove oil (Vix et al., 1967). The pressed peanuts are expanded back to their original size in hot water and drained; salt and other flavoring ingredients are then added, prior to oil- or air-roasting. On a laboratory or pilot-plant scale, the partially defatted peanuts can be prepared with up to 80% of the oil removed, but economics favor producing commercial products with 50–60% of oil removed.

Officials of companies interested in partially defatted peanuts have stated that the market potential would be appreciably increased if the shelf life could be improved. A study was therefore undertaken to improve the shelf life by diffusing nitrogen instead of air into the defatted peanuts.

EXPERIMENTAL

Materials

Commercially pressed, dry-blanched Virginia peanuts were used. These partially defatted peanuts contained 6.1% moisture and 29.1% of oil (58.5% of the original oil removed). After roasting, the peanuts, contained 1.6% moisture and 32.6% of oil (54.3% of the original oil removed).

Methods

Moisture, oil and the peroxide value of oil in peanuts were determined by methods of the American Oil Chemists' Society (1972). In determining the peroxide value residual oil was extracted from peanuts with chloroform.

The glass apparatus used for measuring total gas pressures and the oxygen pressure of this gas which comes from the peanut interstices consists of a 500 ml round bottom flask equipped with a vacuum outlet, a manometer and the sensor of an oxygen analyzer. The oxygen sensor was connected to the glass flask through a stainless steel adaptor. After filling the round bottom flask with partially defatted peanuts (159g) and attaching the mercury manometer, the flask was evacuated to an absolute pressure of 5 mm Hg. Gases in the interstices of the peanuts diffused out into the flask and a record was kept of time versus pressure within the flask. Gas pressures were measured at 24°C.

The oil roaster was a gas fired unit with a rating of 165,000 BTU per hour. A perforated cooler with a fan in the bottom rapidly cooled oil-roasted peanuts by pulling air through them.

Procedures

Two procedures were used. For each test, a batch of 800g of pressed raw peanuts was expanded in hot water at about 93°C for 2 min, drained, 4% salt and 0.5% monosodiumglutamate were added and peanuts were roasted in peanut oil for 3.75 min at 163°C. For the nitrogen cooling procedure the hot roasted peanuts were drained immediately and then cooled in a 10-in. diam Büchner funnel to about 29°C by a flow of nitrogen gas up through the funnel at a controlled rate of approximately 17 cu ft/min for 3.5 min. During cooling of each batch, initial temperature of the nitrogen gas, issuing from the cylinder dropped from about 18°C to 2°C. Temperatures of roasted partially defatted peanuts and cooling gases were taken by means of thermocouples and continuously recorded. Samples of the cooled peanuts were placed in fifteen 211 × 400 (2-11/16 in. diam × 4 in. ht) cans (50g/can). Cans were filled by means of a measured dipper directly over the Büchner funnel through which nitrogen was continuously flowing to prevent contact of air with peanuts. The filled cans, with tops loose, were placed in a chamber containing nitrogen under slight pressure. This prevented entrance of air when each can was removed and placed in a vacuum canning machine. Time of transfer of can (with a lid on top) from chamber to vacuum machine was 3.5 sec. The head space in each can was tested at 24°C for total gas pressure and for oxygen by means of a head-space sampler in conjunction with the oxygen analyzer. The percentage of oxygen in a gas sample was determined from the partial pressure of the oxygen.

The air cooling procedure was comparable to the nitrogen cooling procedure with the exception that air was used in place of nitrogen in the cooling process prior to holding under nitrogen. The perforated fan cooler was used.

Organoleptic tests were conducted on each material at 1, 2, 3, 6, 9 and 12-month intervals. In each test a single can was evaluated by a panel which averaged six members. Results reported are the averages of the individual ratings.

To obtain data for the diffusion of air into the nitrogen-cooled and air-cooled peanuts, peanut samples were exposed to air for various lengths of time immediately after cooling and prior to vacuum canning. Then after equilibrating for at least 48 hr, the head space in each

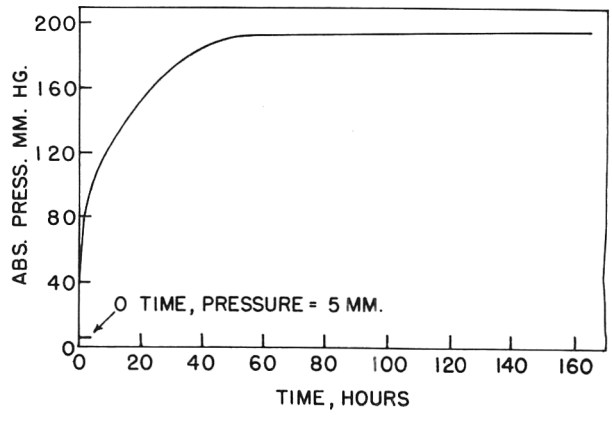

Fig. 1—Pressure in glass flask vs time in flask for defatted peanuts.

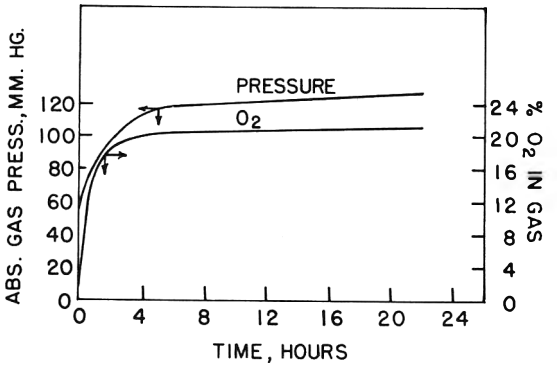

Fig. 2–Diffusion of air into nitrogen-cooled peanuts. Pressure in can vs time of exposure of defatted peanuts to air before canning.

can was tested for gas pressure and oxygen. The oxygen present in the head space is an indication of the air that had been absorbed or diffused into the peanuts during the exposure to air prior to canning.

RESULTS & DISCUSSIONS

FIGURE 1 shows total presure vs time for defatted roasted peanuts that had been cooled and kept in an air atmosphere for 24 hr. These data were obtained with the glass apparatus filled with partially defatted peanuts. At zero time there was an absolute pressure of 5 mm and at the end of 50 hr the absolute pressure generated by the gas diffusing from the interstices out into the space between the peanuts was 190 mm Hg, and finally leveled off at about 200 mm Hg. Apparently a large amount of air was present within the defatted peanuts. This phenomenon shows the defatted peanuts to contain interstices. Interstices are caused by removal of oil and expansion of the peanuts back to their original or greater than original volume.

Figure 2 shows the amount of air (oxygen) that had diffused into nitrogen-cooled peanuts. At the end of 5 hr exposure to air a total of approximately 120 mm Hg pressure was obtained at which time the gas had an oxygen content of 20%. The maximum pressure obtained here is less than that shown in Figure 1, because the cans did not contain as many peanuts per unit volume. Application of the gas laws showed that the gas pressure in Figure 2 was comparable to the 200 mm in Figure 1 when differences in peanuts per unit volume were considered.

Figure 3 is plotted from the data obtained for the first 30 min shown in Figure 2, but on an expanded scale. This type of data can be useful in determining processing conditions. As shown here, at 0 time there was a total gas pressure of about 55 mm Hg of which 1.4 mm was due to oxygen. This is equivalent to an oxygen content of 2.5%. In 5 min the total gas pressure was 65 mm Hg of which 8% was due to oxygen.

Figure 4 shows the diffusion of air (oxygen) into air-cooled peanuts. The total minimum time the peanuts were exposed to air was 5 min, which included the time for air-cooling plus the time for placing the peanuts into the cans. At 5 min there was an absolute gas pressure of approximately 64 mm Hg of which 11 mm (approximately 17%) was due to oxygen. This type data allows evaluation of the effects of the total oxygen content vs shelf life. At 10 min there was an absolute gas pressure of about 75 mm Hg of which 17.5% was due to oxygen. Since the rate of diffusion of air into air-cooled peanuts was slow this suggested the possibility of air-cooling peanuts quickly and holding them in an atmosphere of nitrogen until they could be canned. In addition the low values for oxygen (17 and 17.5%) indicate that nitrogen diffuses faster into the defatted peanuts than oxygen.

Figure 5 shows two possible methods of placing nitrogen or any other inert gas into the interstices of oil-roasted, defatted peanuts. In process I, oil-roast the defatted peanuts at 163°C, cool with nitrogen within 3.5 min, and hold under nitrogen until canned under vacuum at 29.9 in. Hg (absolute pressure 2.5 mm Hg). This would be the more expensive procedure since special equipment would be necessary to cool with nitrogen. In process II, oil-roast defatted peanuts at 163°C, cool with air within 3.5 min, then hold under nitrogen until canned under vacuum at 29.9 in. Hg. Economically, the latter process is more desirable.

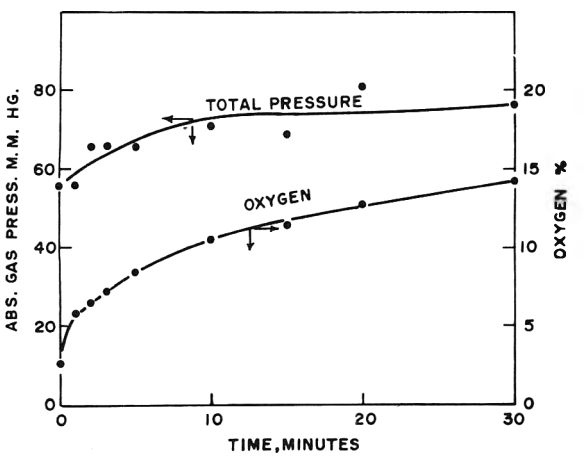

Fig. 3–Diffusion of air into nitrogen-cooled peanuts. Pressure in can vs time of exposure of defatted peanuts to air before canning.

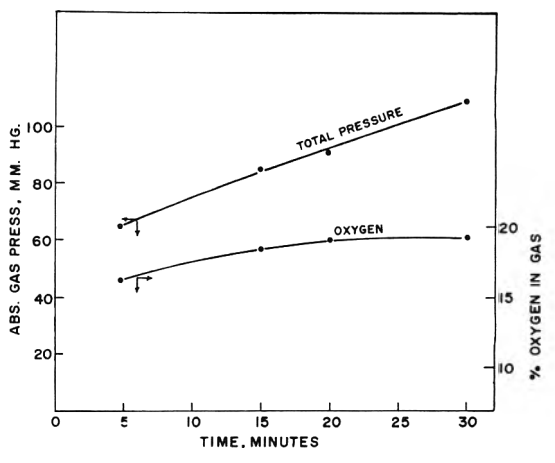

Fig. 4–Diffusion of air into air-cooled peanuts. Pressure in can vs time of exposure of defatted peanuts to air before canning.

Table 1—Effects of nitrogen gas in interstices of partially defatted peanuts on peroxide values during storage at 24°C

Conditions prior to vacuum packaging	Peroxide values meq/kg		
	1 Month	4 Months	12 Months
Air cool, air hold[a]	28	29	19
Air cool, nitrogen hold	4	< 3	3
Nitrogen cool, nitrogen hold	< 3	< 3	< 3

[a] Peroxide value immediately after roasting

Table 2—Effects of nitrogen gas in interstices of partially defatted peanuts on flavor and odor during storage at 24°C

Conditions prior to vacuum packaging	12 months	
	Flavor[a]	Odor[b]
Air cool, air hold	5.8	Off
Air cool, nitrogen hold	6.8	Good
Nitrogen cool, nitrogen hold	7.3	Good

[a] Based on hedonic scale of 9 to 1
[b] Odor when can first opened

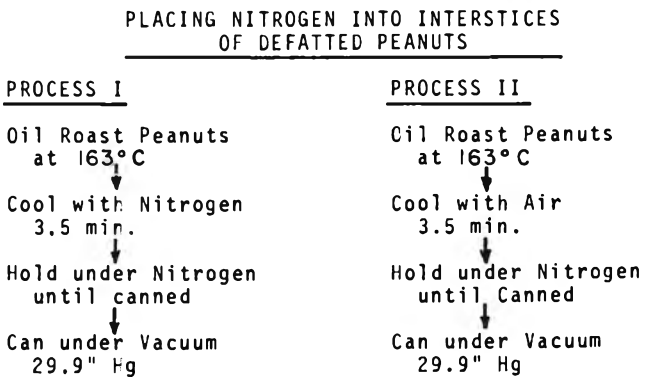

Fig. 5—Two possible methods of placing nitrogen or any other inert gas into the interstices of oil-roasted, defatted peanuts.

Table 1 shows the effect of nitrogen gas in the interstices of partially defatted peanuts on peroxide values of the oil in the peanuts during storage at 24°C. Treatments for the three sets of storage samples were: (1) Air cool, air hold; (2) air cool, nitrogen hold; and (3) nitrogen cool, nitrogen hold. After roasting, the initial peroxide value of the air cool, air hold peanuts was less than three. At the end of 1 month, the peroxide value of the air cool, air hold sample was 28 as compared to 4 and less than 3, respectively, for air cool, nitrogen hold and nitrogen cool, nitrogen hold. These values were about the same at the end of 4 months. At the end of 12 months these values were 19, 3 and less than 3, respectively. Use of either of the latter two methods considerably reduces the peroxide value. Peroxide value is not an exact measure for determining shelf-life storage but it is accepted as an indication of deterioration.

The stored samples were also evaluated for flavor and odor. Results are given in Table 2. Flavor ratings of peanuts at the end of 12 months were 5.8 for air cool, air hold; 6.8 for air cool, nitrogen hold; and 7.3 for nitrogen cool, nitrogen hold.

Flavor values are based on a hedonic scale of 9 to 1. At the end of 12 months the odors upon first opening the cans were respectively off, good, and good. Organoleptic ratings were higher for the two sets of defatted peanuts with nitrogen in the interstices.

SUMMARY

METHODS were developed for improving the shelf life of partially defatted peanuts. Partially defatted peanuts stored under vacuum (5 mm Hg) in a closed container equilibrated to about 200 mm Hg absolute pressure, which was caused by diffusion of air from within the interstices of the peanuts. The air contained oxygen which has a deleterious effect on shelf life of the peanuts. Two procedures were devised for introducing nitrogen into the porous interstices of the defatted peanuts after roasting: (1) cooling oil-roasted defatted peanuts in nitrogen and holding the peanuts under nitrogen until canned under vacuum; or (2) cooling the oil-roasted defatted peanuts in air about 3.5 min and holding the peanuts under nitrogen until canned under vacuum. The latter procedure would be the more practical and economical to use. Shelf-life studies showed that partially defatted peanuts with nitrogen in the interstices have longer shelf life than those containing air.

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RESEARCH NOTES

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A Research Note

EFFECT OF LOW LEVEL GAMMA IRRADIATION ON GROWTH AND PATULIN PRODUCTION BY Penicillium patulum

INTRODUCTION

LOW DOSE gamma irradiation has potential to extend the shelf-life of certain foods (Hannesson, 1972). Hartung et al. (1973) applied a 50 Krad dose to flour and to bread made from the flour and obtained a reduction in the amount of visible and total mold that developed on the bread during storage up to 20 wk. However, a small percentage of molds survived and were capable of outgrowth during storage. The predominate molds found were species of *Aspergillus* and *Penicillium*.

Several workers have reported that gamma irradiation doses below 200 Krad may induce or increase aflatoxin production by *A. flavus* (Jemmali and Guilbot, 1969; 1970a, b; Applegate and Chipley, 1973a, b; Schindler and Noble, 1970). However, other reports show no stimulation of aflatoxin production by this same irradiation dose range in either *A. flavus* or *A. parasiticus* (Schindler et al., 1972; Bullerman and Hartung, 1974). These discrepancies may be due to strain and species differences.

If low level gamma irradiation is to be considered as a potential method of food processing, more information is needed concerning the effects of irradiation on microorganisms important to the public health, including mycotoxin-producing molds. Aside from the work with aflatoxins, little is known of the effects of low level gamma irradiation on molds capable of producing other mycotoxins. Applegate and Chipley (1974) reported increased ochratoxin production by *Aspergillus ochraceus* after irradiation at 150 and 200 Krads. The work reported herein was initiated to determine the effects of low level gamma irradiation on growth and patulin production by strains of *Penicillium patulum*. This organism had previously been isolated from irradiated bread raising concerns about possible effects of irradiation on patulin production in bread (Hartung et al. 1973).

EXPERIMENTAL

ALIQUOTS of 50 ml of potato dextrose broth (pH 5.6) prepared according to the method of Norstadt and McCalla (1969), were used to support growth and patulin production by *P. patulum* in still cultures in 250 ml Erlenmeyer flasks before and after irradiation. Two strains of *P. patulum* were used: strain M108 isolated in our laboratory from irradiated bread; and strain NRRL 989 obtained from the culture collection of the USDA Northern Regional Research Lab. ARS, Peoria, Ill.

Spore suspensions in sterile 0.01% Tween 80 were prepared from 10-day old potato dextrose slant cultures of the organism as previously described (Bullerman and Hartung, 1974). Aliquots of the spore suspensions were irradiated at 0, 100 and 200 Krad at ambient temperature (ca 25°C) using a Cobalt 60 source similar to the one described by Tenny and Miyauchi (1970). Dosimetry for irradiating the inocula was established using the Fricke Dosimeter, ASTMD1671-63. The irradiated and nonirradiated spore suspensions were used to inoculate the flasks of potato dextrose broth in amounts of approximately 10^6 spores per flask. Growing vegetative mycelia, on potato dextrose slants, were also irradiated at the same dosage levels as the spore suspensions. Portions of the mycelia were used to inoculate potato dextrose broth. All treatments were done in duplicate, and the study was replicated three times.

All cultures were incubated at 25°C for 7 days and then given a brief heat treatment (121°C for 30 sec) to kill spores and vegetative mycelia. The mold mats were collected, washed with distilled water, dried at 130°C for 2 hr and weighed. Mold growth was expressed as mg mycelial dry weight. The pH of the filtered broth was determined.

Patulin was extracted from the broth using ethyl acetate in two liquid-liquid extractions of 50 ml each. The extracts were concentrated and the patulin concentration was estimated by visual comparison of samples to known amounts of patulin on thin-layer chromatography (TLC) plates (20 × 20 cm, coated with a 0.25 mm thick layer of Silica Gel (G-HR, Brinkmann Instruments). The plates were developed in toluene/ethyl acetate/formic acid (60/30/10). Patulin was observed as a light-blue fluorescent spot under long wave UV light after exposure to ammonia fumes, and as a yellow colored derivative in natural light after spraying the plates with 4% phenylhydrazine in water and heating for 3 min at 110°C (Scott and Somers, 1968).

Table 1—Production of patulin by Penicillium patulum NRRL 989 and M108 on potato dextrose broth in 7 days of incubation at 25°C when grown from irradiated spores

Irradiation level (Krad)	NRRL 989		M108	
	Broth (μg/ml)	Dry Mycelia (μ/mg)	Broth (μg/ml)	Dry mycelia (μg/mg)
	Patulin			
0 (Control)	137	24	376	67
100	88	19	122	26
200	0.2	0.09	2	0.6

Table 2—Production of patulin by Penicillium patulum NRRL 989 and M108 on potato dextrose broth in 7 days of incubation at 25°C when grown from irradiated vegetative mycelia

Irradiation level (Krad)	NRRL 989 Culture broth (μg/ml)	NRRL 989 Dry mycelia (μg/mg)	M108 Culture broth (μg/ml)	M108 Dry mycelia (μg/mg)
	Patulin			
0 (Control)	32	9	13	3
100	ND[a]	–	0.7	0.2
200	ND[a]	–	ND[a]	–

[a] ND = None cetected.

RESULTS & DISCUSSION

Growth

Low level gamma irradiation of spores of *P. patulum* reduced subsequent growth in potato dextrose broth of both strains studied. After 7 days of incubation, the growth of strain NRRL 989 from spores irradiated at 100 Krad was 81% of the control. Growth from spores irradiated at 200 Krad was 40% of the control. Strain M108 was somewhat more resistant to irradiation than strain NRRL 989. Growth of strain M108 from spores irradiated at 100 Krad and 200 Krad was 84 and 65% of the control, respectively. Irradiation of growing vegetative mycelia resulted in variable growth by subsequent cultures in potato dextrose broth. The growth of strain NRRL 989 from irradiated mycelia was equal to or greater than the control at both the 100 and 200 Krad doses. The growth of strain M108 was less than the control at the 100 Krad dose, but equal to the control at the 200 Krad dose. The final pH of all control cultures was lower than the final pH of cultures grown from irradiated inocula.

Patulin production

Production of patulin by cultures grown from irradiated spores was substantially less than the control cultures (Table 1). Strain NRRL 989 produced 36% less patulin after irradiation at 100 Krads and 99+ % less after irradiation at 200 Krads. Strain M108 produced 68% less patulin at 100 Krads and 99+ % less at the 200 Krads dose. Patulin production by cultures grown from irradiated vegetative mycelia produced even less patulin than cultures from irradiated spore cultures (Table 2). Strain NRRL 989 did not produce detectable amounts of patulin after either irradiation treatment of mycelia and strain M108 produced trace amounts of patulin after irradiation of mycelia at 100 Krads.

There was no stimulation of patulin production observed in strain NRRL 989 or M108 from spores or mycelia by either level of irradiation. The amount of patulin produced per mg of dry mycelia declined steadily as the irradiation dosage increased. Complete inhibition of growth or patulin production was not achieved by irradiation of spores at levels up to 200 Krads. Irradiation of mycelia likewise did not eliminate growth, but did eliminate patulin production at the 200 Krad dose level.

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A Research Note
EFFECT OF IRRADIATION ON VOLATILE CONSTITUENTS OF STORED HADDOCK FLESH

INTRODUCTION

IN RECENT YEARS the extension of shelf life of sea foods has received a great deal of consideration both in the United States and in many other countries of the world. In order to meet the world's growing need for fresh protein, even greater stability is required than refrigeration alone can provide. Since many commercial food fishes are taken from relatively cold waters, spoilage due to autolytic processes and microbial contamination of these food fishes continues even at cool temperatures. Thus the effectiveness of refrigeration for shelf life extension is reduced. It is most noticeable in the flavor of fresh fish, its most perishable characteristic.

Flavor deterioration in fish during storage is believed due primarily to microbial growth. Mendelsohn et al. (1966) have shown that the volatile compounds of stored haddock fillets increased with storage time and decreasing organoleptic quality. It would therefore be expected that objectionable flavor changes in fish could be diminished by Υ-irradiation treatments that would materially reduce the microbial population in fish, especially since the active spoilage organisms are very susceptible to Υ-rays. However, undesirable flavor changes in fish may be brought about by sterilizing doses of irradiation (Mendelsohn and Brooke, 1968).

Several other researchers have studies the effects of the irradiation process on sea foods. The effects of radiation on clam meats were studied by Gadbois et al. (1967) for development of carbonyl compounds and by Mendelsohn and Brooke (1968) for volatile compounds in the headspace vapors. Trimethylamine and amino nitrogen content measurements were made by Chung (1963). Novak and Luizzo (1963) studied the volatile fatty acids, carbonyl compounds and amino compounds in irradiated shrimp. Chemical changes attributed to low doses of radiation (radio-pasteurization) were found in all of these studies. Mendelsohn et al. (1969) have also shown, using gas chromatography, that both the number and the concentrations of volatile compounds isolated and identified from haddock increased with increasing radiation dose. Nevertheless, Connors and Steinberg (1966) have shown organoleptic acceptability of haddock fillets irradiated at 150 and 250 Krad and stored from 0–30 days at $\approx$1°C. They also found that irradiation flavors and odors were not detected in any of the taste tests.

The purpose of this investigation was to study the effects of irradiation and storage on haddock fillets by analyzing the volatile constituents.

EXPERIMENTAL

FRESH HADDOCK FILLETS, obtained from a local dealer, were placed in mylar bags, sealed and irradiated at doses of 0, 0.2, 2.8 and 5.6 Mrad while keeping the fillets at 0–5°C. Samples from all treatments were taken immediately after processing. After storage at 5.5°C for 14 days, samples were taken of the fish treated at 0 and 0.2 Mrad. Samples of the fish treated at 0.2, 2.8 and 5.6 Mrad were taken after 30 days storage at 5.5°C.

Duplicate 1200-g samples of minced haddock flesh were prepared. The total condensate was collected from one of the 600g series (neutral) of the samples by low temperature-high vacuum distillation (Merritt et al., 1959) without further treatment. The remaining 600-g portions of each sample, forming a second series (alkaline), was first treated by adding 60 ml of 6N sodium hydroxide to the bottle, followed by adding 60 ml of distilled water and again mixing by shaking the bottle before collection of the total condensate in the same manner as for the first (neutral) 600g series of sample portions.

A "center fraction" (Merritt et al., 1959), or that fraction of the total condensate containing compounds exerting vapor pressure between the temperatures of −140°C and −80°C (most of CO_2 and H_2O removed), was separated from each of the total condensates obtained by low temperature-high vacuum distillation. All of these center fractions were analyzed by a combined programmed temperature gas chromatography and mass spectrometry instrument system (Merritt et al., 1966).

The components in the center fractions from the neutral sample portions were separated on a 50 ft × 0.02 in. i.d. stainless steel support coated open tubular column with 1,2,3, tris (cyanoethoxy) propane as the stationary phase and a helium carrier gas flow rate of 5 ml per minute. Separation of the components in the center fractions from the alkaline treated sample portions was achieved with a 10 ft × 1/8 in. o.d. stainless steel column packed with 60–80 mesh chromasorb 103 coated with 10% carbowax 20M plus 3% KOH and a helium carrier gas flow rate of 20 ml per minute. The effluent of the gas chromatograph was eluted directly into the source of a Bendix Model 14 time-of-flight mass spectrometer. This mass spectrometer was equipped with several output devices including: a strip chart recorder to record the gas chromatogram of the sample by monitoring the total ion current of the mass spectrometer; a digital integrator for measuring the printing out both the area and retention time of each gas chromatographic peak; an oscilloscope for displaying the mass spectra; and a recording oscillograph for recording the mass spectra of each gas chromatographic peak as it elutes from the column. This output system made available both qualitative and quantitative data (Merritt, 1970).

RESULTS & DISCUSSION

THE COMPOUNDS found in both fresh and stored, irradiated and nonirradiated (neutral) haddock sample protions are listed in Table 1. These results show a few major differences between irradiated and nonirradiated samples. In general, more compounds were isolated from the irradiated samples than were isolated from the nonirradiated samples. This is expected, since irradiation causes many microchemical reactions in the fish flesh producing a wide variety of volatile compounds (Mendelsohn et al., 1969). Also, the irradiated samples contain more hydrocarbons. Few compounds were found in the sodium hydroxide treated samples. These compounds were methylamine, trimethylamine, dimethyl sulfide, methanol and ethanol; and they were found in all basic sample portions. The compound identities were the same for all irradiated samples as well as for all nonirradiated samples but varied greatly in amounts depending on irradiation dose and time in storage.

The overall results, reflecting the effects of radiation and storage on haddock fillets, combining the results of both the

neutral and basic sample portion series, are summarized in Figure 1. This figure shows only those classes of compounds that were found in large enough amounts in each sample to depict graphically. Amines, predominantly trimethylamine, were found in all basic sample portions regardless of storage time or radiation dose, even though the unstored haddock was judged to be fresh fish. This finding was true only for the basic sample portions and not for the neutral ones, indicating a release of trimethylamine by the addition of sodium hydroxide to the haddock flesh. Trimethylamine was found in the neutral sample portions only for the nonirradiated samples stored for 14 days. Nevertheless, before storage, higher concentrations of compounds were found, mostly hydrocarbons and sulfur compounds, in the irradiated haddock, the amounts being proportional to the irradiation dose. However, after storage, the nonirradiated samples contained the larger amounts of compounds, which are mainly sulfur and amine compounds with smaller amounts of carbonyl compounds and alcohols. It is also noticed that on storage, the hydrocarbons and other compounds formed by irradiation diminish in quantity, especially the more volatile ones. This is believed due to the permeability of these gases through the mylar package. It can also be seen that the samples treated with the lower irradiation doses formed sulfur and amine compounds on storage, but not to the extent of the nonirradiated samples.

Table 1—Compounds identified from neutral sample portions of irradiated and nonirradiated haddock fillets

Irradiated		Non-Irradiated
butane	ethanal	ethanal
butene-1	2-me-propanal	2-me-propanal
pentane	butanal	butanal
pentene-1	2-me-butanal	2-me-butanal
methylpentane	acetone	acetone
hexane	butanone-2	methanol
hexene-1	methanol	ethanol
heptane	ethanol	ethyl mercaptan
heptene-1	ethyl mercaptan	dimethyl sulfide
octane	dimethyl sulfide	dimethyl disulfide
benzene	dimethyl disulfide	methylamine
toluene		trimethylamine
		benzene
		toluene

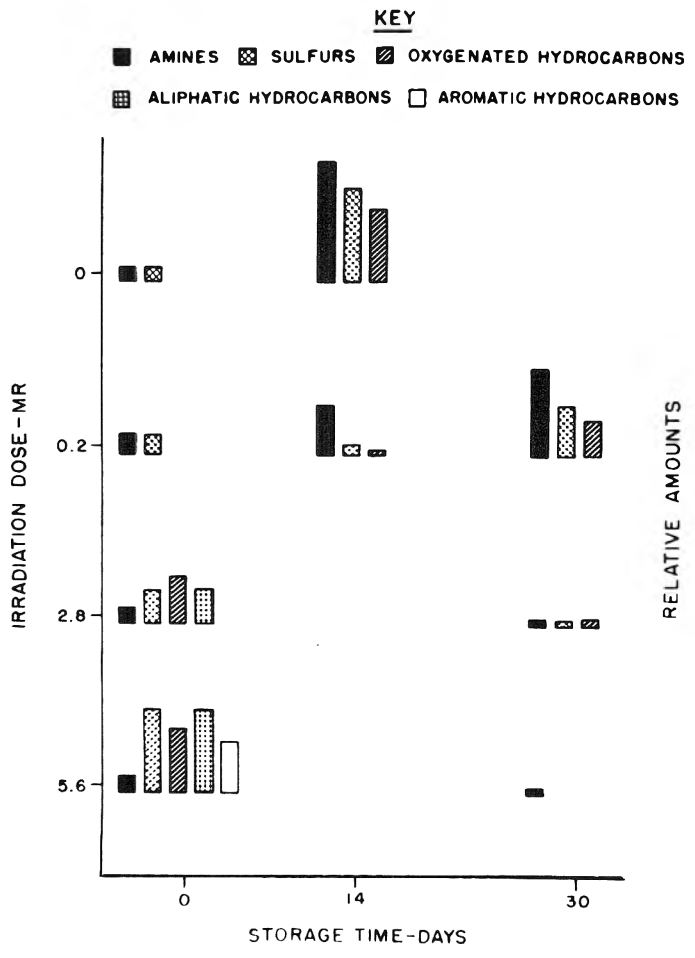

Fig. 1—Relative amounts of classes of volatile compounds found in stored haddock fillets.

Haddock irradiated at 0.2 Mrad shows an increase in volatile material with storage time although the increase is smaller than the nonirradiated haddock samples. This clearly indicates that although the microbial population is vastly decreased by this low irradiation dose, the haddock flesh is by no means sterile. The sterilized sample (5.6 Mrad) shows a sharp decrease in volatile compounds on storage. Samples irradiated at 2.8 Mrad also show a decrease in volatile compounds during storage although not as sharply as when irradiated at 5.6 Mrad. This indicates that although 2.8 Mrad is not a high enough dose for sterilization, it has decreased the microbial activity to such an extent that the overall losses of volatile compounds in this dynamic system exceed microbial production of volatile constituents during storage.

Sensory evaluations were not performed in this study. However, on comparing the processing and storage conditions in this study with those in studies where sensory evaluations were performed, some interesting correlations may be drawn. Since Connors and Steinberg (1966) stated that the taste panel used in that study did not indicate the presence of irradiation flavors or odors in any of the samples, which included doses as high as 0.35 Mrad; the unstored samples irradiated at 0.2 Mrad in this study would not be expected to possess an irradiated flavor. Connors and Steinberg (1966) have also shown that haddock fillets irradiated at 0.25 Mrad were still organoleptically acceptable after 30 days in storage at 1°C, although acceptability decreased after 20 days in storage. Since the irradiation dose and storage condi-

tions for the study by Connors and Steinberg (1966) are similar to those in this study for the 0.2 Mrad irradiated samples, it would be expected that the extent of the increase in concentrations of volatile compounds found after 14 days of storage would indicate no decrease in organoleptic acceptability, whereas the extent of the increase in concentrations of volatiles in the samples stored for 30 days would indicate a decrease in organoleptic acceptability.

Ronsivalli et al. (1968) point out the pronounced organoleptic changes brought about in fish by sterilization doses of ionizing radiations. Such changes would be expected in the haddock fillets sterilized by irradiation with a dose of 5.6 Mrad in this study. This is consistent with the relatively high concentration of volatiles, especially sulfur and carbonyl compounds found for the unstored haddock fillets irradiated at 5.6 Mrad in this study. Although the concentration of volatiles in the haddock fillets irradiated at 2.8 Mrad was considerably lower than in the samples irradiated at 5.6 Mrad before storage, they probably contained a high enough concentration of volatile compounds to alter the organoleptic quality of the fillets. However, after 30 days in storage under the conditions used in this study, the samples irradiated at 5.6 Mrad and 2.8 Mrad both contained lower concentrations of volatile compounds than did the samples irradiated at 0.2 Mrad. In view of these findings it would be expected that the samples analyzed in this study irradiated at 2.8 Mrad and perhaps even those irradiated at 5.6 Mrad at 5°C and stored at refrigerated temperature could have good organoleptic quality. Mendelsohn et al. (1970) also found cod fillets to be organoleptically acceptable after irradiation at 4.5 Mrad at cryogenic temperatures. Although it must be kept in mind that production of volatile compounds is not the only change affecting organoleptic quality that would be initiated by ionizing radiation, it can serve, however, as a good indicator for the extent of change which has occurred.

CONCLUSIONS

CONSIDERING these results and related studies (see references) it may be concluded that:

1. The microbial deterioration of chill-sotred haddock can be reduced by irradiation which decreases the spoilage microorganisms and thereby the shelf life of haddock is increased.

2. If an odor is caused by volatiles formed in irradiated haddock, it probably decreases with storage time if the product is packed in a gas permeable container.

3. The types and overall patterns of compounds produced by irradiation and microbial deterioration of fish are quite similar but differ significantly with respect to hydrocarbons and amines.

4. The expected shelf life at a given storage temperature would depend on the irradiation dose.

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A Research Note
EFFECT OF TEMPERATURE AND HEATING TIME ON THE DETECTION OF OFF-FLAVOR IN AVOCADO PASTE

INTRODUCTION

THERMAL PROCESSING of avocado paste is problematic due to the appearance of off-flavor when avocado pulp is heated above a certain temperature. This off-flavor is caused by a combination of several chemical substances, some of them already present in the avocado pulp and others formed during heating (Bates, 1970).

The appearance of off-flavor can be detected by sensory tests after processing at several temperatures and times, to construct a curve that could be used in combination with a thermal death time (TDT) curve, for a suitable microorganism like *Bacillus coagulans*, to select adequate times and temperatures for a High Temperature Short-Time (HT-ST) process for avocado paste acidified with lime juice.

EXPERIMENTAL

A BATCH of avocado paste was prepared from avocados of the Aztec variety, all harvested on the same orchard. The fruits were allowed to ripen and the paste was then prepared by thoroughly mixing the pulp with the ingredients in the proportions shown in Table 1. The batch was frozen into a number of plastic bags, each containing 284g of paste. For each thermal process test, the contents of a bag were thawed and filled into small 28g capacity, 8 X 10 cm, low density polyethylene bags, which were heat sealed and then immersed in a controlled temperature water bath during a specified time, after which they were cooled with running cold water.

After cooling, a sensory evaluation was performed in a triangular test in comparison with an unprocessed control sample from the same batch (Larmond, 1970).

A selected and trained panel of 10 persons was used for the sensory test. If no significant differences were found at the α=0.05 level, a longer process time at the same temperature was tested.

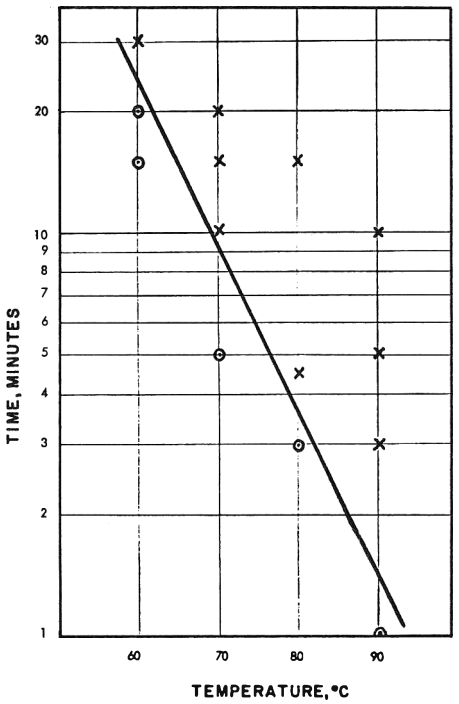

Fig. 1—Effect of temperature and heating time on the appearance of off-flavor in avocado paste.

Table 1—Avocado paste formula

Ingredient	%
Sodium chloride	1.43
Lime juice	4.60
Wild marjoram powder	0.10
Onion powder	0.27
Sodium bisulfite	0.03
Sorbic acid	0.025

RESULTS & DISCUSSION

FIGURE 1 is a curve for the detection of off-flavor in avocado paste in which process times were plotted against temperature. Round points mean no significant difference, while crosses represent samples in which off-flavor was detected.

The procedure was similar to that of a TDT curve. A preliminary z value of 24.26 was calculated. Processing times shorter than 5 min are still not corrected for heating lag. Corrections should be determined experimentally for avocado paste. Corrected times will give a smaller z value.

For better usefulness, data on the appearance of off-flavor at higher temperatures and shorter processing times are needed. A stronger packaging material should be used for the experiments, because low density polyethylene failed at temperatures higher than 90°C.

To establish an adequate thermal process, a TDT curve for a suitable microorganism in avocado paste should be constructed. *Bacillus coagulans* is suggested, because it has fairly heat-resistant spores and can grow in an acid food such as avocado paste acidified with lime juice (pH = 4.0).

The small z value obtained from the curve on Figure 1 is inconvenient because it is similar to typical z values for microorganisms (Leonard, 1973).

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A Research Note

POLYGALACTURONASE ACTIVITY IN CITRUS FRUIT

INTRODUCTION

STRUCTURAL changes of pectic substances, caused by pectic enzymes, are an important factor in the ripening of fruits and vegetables and in the consistency and stability of their products. Citrus fruit is rich in pectic substances, but until now it was not clear whether it contains pectinases, and especially polygalacturonase (PG). Several workers (MacDonnel et al., 1945; Mannheim and Siv, 1969; Korner, 1971) failed to find PG activity in oranges, lemons and mandarins. In grapefruit, however, a slight PG activity was detected (Pratt and Powers, 1953; Mannheim and Siv, 1969). The failure to demonstrate clearly the presence of PG in citrus, led to the assumption that destruction of pectic substances in citrus tissues is due to pectin esterase activity followed by nonenzymatic reactions (Joslyn and Pilnik, 1961).

It has been reported (Cook et al., 1970; Albersheim and Anderson, 1971) that plant tissues contain inhibitors of PG. The possibility that the failure to find PG activity in citrus fruit could be caused by the presence of inhibitors was tested by Mannheim and Siv (1969), but they did not observe any inhibition of tomato PG by citrus extracts. These data are in contrast to other reports (Cole and Wood, 1970; Rogers and Hurley, 1971) indicating the presence of PG inhibitors in citrus fruit.

Recently we isolated a PG from abscission zones of leaves (Riov, 1974) and fruit (Greenberg, Goren and Riov, unpublished) of citrus. PG activity increased during and following abscission of these organs and this increase was accelerated by ethylene. Determination of PG activity required the inhibition of an enzyme, named by us uronic acid oxidase, which was present in the extracts used. This enzyme oxidizes the reaction products of the PG as well as the free reducing groups of the substrate.

Based on our recent work and on data in the literature, the present work was designed to study the possible factors which might interfere with the determination of PG in citrus fruit tissues and to determine whether PG occurs in citrus fruit.

EXPERIMENTAL

Source of fruit

Ripe Shamouti and Valencia oranges, Marsh Seedless grapefruit and lemons were harvested from 35 to 40-yr-old trees, grown in commercial groves. Fruit was tested on the day of picking.

Isolation of citrus fruit PG

12-g samples of fruit tissues were homogenized with an Ultra Turrax homogenizer in 120 ml of 50 mM potassium phosphate buffer, pH 7.5, containing 6% (w/v) $(NH_4)_2SO_4$. All the extraction and concentration steps were carried out at 0–4°C. The homogenate was stirred for 30 min, filtered through a nylon fabric and centrifuged for 10 min at 20,000 X G. The PG was precipitated from the supernatant by slowly adding $(NH_4)_2SO_4$ up to 80% saturation, letting the mixture stand for 1 hr and centrifuging for 10 min at 20,000 X G. The supernatant fraction was discarded and the pellet was dissolved in 8 ml of 1% NaCl and dialyzed against two changes of 1% NaCl. The dialyzate was clarified by centrifugation and brought to a constant volume with 1% NaCl.

PG assay

Determination of PG activity was usually performed by measuring liberation of reducing groups. The standard 1-ml reaction mixture contained 0.5 ml enzyme solution, 0.25% sodium polypectate (Sigma Chemical Co.) and 1 mM sodium hydrosulfite in 50 mM sodium acetate buffer, pH 5.0. A heated enzyme blank was run with each experiment. Reaction mixtures were incubated at 37°C for 24 hr. The increase in reducing groups was measured with the dinitrosalicylic acid (Merck AG) reagent (Miller, 1959), using galacturonic acid as a standard. One unit of enzyme activity is defined as that amount of enzyme which catalyzes the liberation of 1 μmole galacturonic acid in 24 hr under the specified reaction conditions.

Table 1—Polygalacturonase activity in citrus fruit tissues

Fruit	Polygalacturonase activity (units/g fresh wt)		
	Flavedo	Albedo	Pulp
Shamouti orange	1.23	0.69	0.41
Valencia orange	1.55	0.85	0.23
Grapefruit	1.70	1.85	0.34
Lemon	1.26	0.52	0.15

PG activity was also measured viscosimetrically. The standard reaction mixture in a total volume of 7 ml contained 0.5 ml enzyme solution, 1.2% sodium polypectate and 1 mM sodium hydrosulfite in 50 mM sodium acetate buffer, pH 5.0. The change in viscosity was determined after incubation at 37°C for 24 hr with Cannon 200 viscosimeter.

Inhibition of pectinase by citrus extracts

The possible presence of PG inhibitors in citrus fruit was tested in three different preparations: centrifuged juice; the PG preparations of flavedo, albedo and pulp described above; and crude extracts of rind and pulp. The crude extracts were prepared by homogenizing 10g of tissue in 100 ml of 50 mM phosphate buffer, pH 7.5, containing 5% (w/v) NaCl and centrifuging for 10 min at 20,000 X G.

Samples of 0.9 ml of each of the above preparations were added to 0.1 ml of pectinase solutions obtained from the following sources: fungal pectinase (Sigma Chemical Co.); tomato PG isolated by the method of Patel and Phaff (1960); a dialyzed filtrate from a 7-day-old *Penicillium digitatum* culture grown on a medium described by Cole and Wood (1970). After preincubation for 10 min, the effect of the citrus preparations on pectinase activity was measured viscosimetrically.

Ethylene treatment

Fruit was treated with ethylene as previously described by Riov et al. (1969).

Results are the averages of three independently prepared samples, six fruits per sample. Enzymatic assays were done in duplicate.

RESULTS & DISCUSSION

TESTING the various preparations obtained from citrus fruit, we were unable to detect any inhibitory effect of citrus extracts on pectinase activity. These results are in accordance with the observations of Mannheim and Siv (1969). Although other reports (Cole and Wood, 1970; Rogers and Hurley, 1971) indicate the presence of PG inhibitors in citrus fruit, our data show that the PG preparations used by us were free from inhibitors.

Flavedo and albedo preparations used for the determination of PG activity contained uronic acid oxidase activity, assayed by the disappearance of galacturonic acid from the reaction mixtures (Riov, 1974). No significant uronic acid

oxidase activity was found in pulp preparations. It is, however, possible that uronic acid oxidase occurs also in pulp but its activity is too low to be measured by the method used. Inhibition of uronic acid oxidase was, therefore, needed, at least in flavedo and albedo extracts, prior to the determination of PG activity. This was achieved by adding 1 mM sodium hydrosulfite to the reaction mixtures (Riov, 1974).

After the problems involved in the determination of PG in citrus fruit had been studied, at least in part, we determined the PG activity in mature citrus fruit tissues (Table 1). PG activity was found in flavedo, albedo and pulp of Shamouti and Valencia oranges, grapefruit and lemons. Except for grapefruit, the highest activity on fresh weight basis, was found in the flavedo and the lowest activity in the pulp. In grapefruit, the highest activity was found in the albedo. No significant PG activity was detected in the same preparations by viscosimetry. This might be explained by our previous observations indicating that citrus PG is an exo PG (Riov, 1974) and determining its activity by reducing groups liberation is, therefore, much more sensitive than by the change in viscosity. Pectin was also hydrolyzed by the preparations used, but only at about 50% the rate of sodium polypectate.

PG activity in citrus fruit tissues is relatively very low compared to the activity found in the abscission zones of abscising citrus organs and in other various fruits and vegetables. It is unlikely that the very low PG activity accounts for certain processes which occur in citrus fruit products, such as cloud instability of natural juice.

The effect of ethylene on PG activity in citrus fruit tissues was tested by treating Valencia oranges and grapefruit with 10 μl/liter ethylene for 48 hr. Ethylene had no effect on PG activity as compared to air controls. Using a bioassay for measuring pectinase activity, Rogers and Hurley (1971) detected a low level of pectinase activity in rind tissues of oranges which was not affected by ethylene. On the other hand, they found that ethylene increased pectinase activity in the abscission zone of the fruit. We also found that ethylene increased PG activity in the abscission zones of leaves (Riov, 1974) and fruit (Greenberg, Goren and Riov, unpublished) of citrus. From the above data it might be concluded that ethylene affects PG activity in the abscission zones of citrus organs, but has no effect on PG activity in fruit tissues.

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A Research Note

VISCOSITY OF MANGO NECTAR AS RELATED TO PECTIC SUBSTANCES

INTRODUCTION

THE NATURALLY OCCURRING pectic materials in most fruits and vegetables have a marked effect on the texture of both the fresh and processed product. Numerous studies reported in the literature have shown that texture is closely associated with the amount and nature of pectins in the fruit during the final stages of ripening. In peaches the typical melting-fleshed varieties have a relatively high proportion of their pectins in a water soluble form when ripe. In contrast, firm-fleshed varieties show a higher proportion of their pectins to be present in an insoluble form when the optimum stage of maturity is reached (Postlmayr et al., 1956; Shwefelt et al., 1971). Secondly, texture and consistency of jam, nectar or syrup are markedly influenced by the pectins that are originally present in the fruit because of their thickening and jelling properties.

No information is available on the pectic constituents of mangoes as affected by the ripeness level and varietal characteristics. Sanchez-Nieva et al. (1959) stated that because of variation in pectin content in different varieties of mangoes it is impossible to obtain nectars of uniform consistency by dispersing and diluting the pulp in a constant proportion of water by weight. The work reported here was done in an attempt to demonstrate the relationship between harvest date, pectin content and their effect on viscosity of mango nectar.

MATERIALS & METHODS

FRUITS of three mango cultivars—Alphonso, Kitchener and Abu Samaka (similar to Indian Tetapuri)—were obtained from orchards about 25 yr old. Each time, 120 fruits were harvested at intervals of 7–10 days from three trees, placed in ventilated wooden crates and left to ripen in the laboratory (avg temperature 28–30°C, relative humidity 45%). The fruits were inspected daily and the ripe ones were stored at 20°C. Twenty fruits of the hard and of the ripe were used for pectin determination. Pectin was similarly evaluated in the tree-ripened fruits. The experiment was conducted for two consecutive seasons.

For nectar manufacture fully ripe fruits were washed, peeled and sliced using stainless steel knives. The sliced mango was pulped through a pulper using a 0.045-in. screen. Nectar was constituted from 1,000 kg mango pulp,

Table 1—Effect of harvest date on pectin content of mangoes

			Total pectin "Fresh basis" %		Protopectin "Fresh basis" %		Water-soluble pectin "Fresh basis" %	
Variety	Harvest date		Hard	Ripe	Hard	Ripe	Hard	Ripe
Alphonso	Season 1971 –	3 May	0.426	0.370	0.426	0.350	Nil	0.019
		23 May	0.378	0.370	0.377	0.334	–	0.036
		1 July	0.269	0.262	0.269	0.222	trace	0.042
		Tree ripe	–	0.269	–	0.206	–	0.063
	Season 1972 –	30 April	0.510	0.464	0.510	0.464	trace	trace
		22 May	0.315	0.312	0.315	0.240	trace	0.072
		14 June	0.260	0.252	0.260	0.179	trace	0.073
		Tree ripe	–	0.260	–	0.179	–	0.081
Kitchener	Season 1971 –	8 May	0.386	0.380	0.386	0.347	Nil	0.033
		16 May	0.318	0.260	0.306	0.220	0.016	0.040
		Tree ripe	–	0.292	–	0.167	–	0.125
	Season 1972 –	29 April	0.391	0.380	0.391	0.363	Nil	0.017
		13 May	0.345	0.336	0.345	0.245	trace	0.017
		27 May	0.335	0.308	0.293	0.225	0.042	0.083
		Tree ripe	–	0.244	–	0.120	–	0.124
Abu Samaka	Season 1971 –	14 June	0.437	0.420	0.437	0.420	Nil	Nil
		21 June	0.420	0.408	0.420	0.368	Nil	0.040
		1 July	0.440	0.396	0.440	0.330	Nil	0.066
		11 July	0.422	0.320	0.422	0.150	Nil	0.180
		21 July	0.392	0.354	0.392	0.112	Nil	0.242
		Tree ripe	–	0.339	–	0.077	–	0.262
	Season 1972 –	1 July	0.655	0.618	0.638	0.518	trace	0.100
		9 July	0.655	0.382	0.426	0.276	trace	0.106
		30 July	0.450	0.440	0.380	0.328	0.070	0.112
		Tree ripe	–	0.420	–	0.299	–	0.129

Table 2—Effect of harvest date on pectin content and viscosity of canned mango nectar

Variety	Harvest date		Total pectin (g/100g)	Viscosity at 20°C	
				Tube flow time (sec)	Stormer relative viscosity
Alphonso	Season 1971 —	3 May	0.215	22.3	33.0
		25 May	0.171	11.3	8.1
		1 June	0.148	3.9	2.7
		Tree ripe	0.137	3.7	2.7
	Season 1972 —	30 April	0.212	47.3	20.2
		22 May	0.176	17.0	3.2
		14 June	0.164	3.2	15.7
		Tree ripe	0.161	8.38	3.3
Kitchener	Season 1971 —	8 May	0.229	42.5	11.9
		16 May	0.208	5.2	4.7
		Tree ripe	0.198	4.5	3.9
	Season 1972 —	29 April	0.204	34.3	29.8
		13 May	0.200	25.9	22.6
		27 May	0.136	10.6	15.8
		Tree ripe	0.174	9.4	6.2
Abu Samaka	Season 1971 —	14 June	0.211	19.2	15.2
		21 June	0.209	18.6	14.7
		1 July	0.213	19.6	16.1
		11 July	0.214	19.5	15.8
		21 July	0.245	17.4	15.2
		Tree ripe	0.205	19.0	15.2
	Season 1972 —	1 June	0.249	33.0	25.3
		9 July	0.198	22.0	21.6
		30 July	0.232	18.0	17.3
		Tree ripe	0.218	20.0	18.0

1,000 kg water and 0.20 kg sugar. The nectar was heated to 98°C and filled in previously sterilized cans and stored at 7°C until examined.

For analysis the slices obtained from 20 fruits were blended in a Waring Blendor. Viscosity measurements were carried out with a tube and Stormer viscometer at 20°C. A 35 cm long tube, 0.6 cm o.d. and 0.29 i.d., was marked in the middle in two places 21 cm apart. The flow time of the nectar through the 21 cm was recorded as a measure of viscosity. The data reported were an average of five determinations. Stormer readings of mango nectar samples were taken without any dilution. 92 ml of samples were placed in the compartment and the time in seconds for 100 revolutions with a 166g driving weight was recorded. The reading for distilled water at 20°C was 6 sec. Water-soluble pectin was extracted with distilled water and precipitated with 0.5N hydrochloric acid and 95% ethanol. The precipitate was separated by centrifugation washed with 95 and 70% ethanol and then suspended in 0.05N sodium hydroxide and diluted to 50 ml with distilled water. For extraction of total pectin the method described by McCready and McComb (1952) was used. Pectin content was estimated colorimetrically as described by McComb and McCready (1952). Protopectin was calculated from the difference between total and soluble pectin.

RESULTS & DISCUSSION

RESULTS of the total pectin, protopectin and water-soluble pectin are shown in Table 1. Total pectin of hard and ripe fruits showed a steady decrease during the picking seasons for Alphonso and Kitchener. Total pectins from fresh hard or ripe fruits from Abu Samaka showed a fluctuating trend due to the fact that this cultivar matures earlier than the traditional harvest time (Saeed, 1974). The pectin content of Abu Samaka was higher compared to the other two cultivars. After ripening of the fruits from the three cultivars in the laboratory, total pectin decreased. This might be due to degradation of pectic substances to other forms that cannot be precipitated by alcohol used in this method of extraction.

Results in Table 1 show that the water-soluble pectin increased from 0.019 to 0.063 for Alphonso, 0.033 to 0.105 for Kitchener and from nil to 0.26% for Abu Samaka during the 1971 season. Tree-ripe fruits, especially those from Abu Samaka, showed the highest increase in water-soluble pectin. The protopectin content declined as the season advanced.

Results in Table 2 show the effect of picking time on the viscosity of mango nectar. A relationship between harvest date, total pectin and viscosity is borne out. Nectar obtained from early harvests, especially in Alphonso, was high in total pectin and had high viscosity, whereas nectars obtained from late pickings were low in total pectin and viscosity. Viscosity of nectars prepared from Abu Samaka did not show the decreasing trend during the picking season with exception of the first harvest in 1972; this seems to support the finding that this variety matures early. Nectars from Abu Samaka showed higher viscosity than those from the other two cultivars. This was due to the higher pectin content of that cultivar.

Saeed (1974) treated mango nectar with pectic enzyme at different concentrations and then pasteurized the nectar. The viscosity of the nectar dropped appreciably indicating that the pectic substances constitute a major factor contributing to viscosity of mango nectar.

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A Research Note

CHARACTERIZATION OF PECTIC SUBSTANCES IN MANGO MARC

INTRODUCTION

SINCE the nature of the protopectin is unknown, the changes in pectin leading to greater solubility are not fully understood. A correlation between changes in solubility of pectin and changes in solubility of calcium led Doesburg (1957) to suggest that solubilization of pectin during ripening of apples may be caused by movement of calcium in the cell walls in response to changes in pH and organic acids. It is also possible that the insolubility of protopectin is due to a structure consisting of high molecular weight chains which may be associated with each other and other cell wall polysaccharides. Solubilization of this polysaccharide complex would involve enzymatic cleavage of critical bonds, including depolymerization of polygalacturonide chains.

Complete characterization of pectic substances was considered necessary by McComb and McCready (1952) for controlling the texture of fruits and vegetables during processing and storage.

In the present study, pectin fractions from three mango cultivars were characterized for molecular weight, free, acetyl and esterified carboxyl group content and for the anhydrouronic acid content. The purpose was to determine the variations in these characteristics in relation to textural properties of the three mango cultivars.

MATERIAL & METHODS

FRUITS of three mango cultivars–Alphonso, Kitchener and Abu Samaka–were harvested during the peak of the season and ripened in the laboratory (avg temperature 28–30°C, relative humidity 45%). Total pectin was extracted and determined according to the method of McCready and McComb (1952) and McComb and McCready (1952) with slight modification. The marc from each cultivar was obtained from the pulp by extraction first with 95% ethanol, acidified ethanol, 70% ethanol and finally washed with acetone. The marc was dried at room temperature, ground and passed through 40-mesh (Gee et al., 1958).

Free acidity was determined by titration with 0.2N sodium hydroxide using phenol red indicator. Degree of esterification was determined by saponification with 0.5N hydrochloric acid and titrated with 0.2N sodium hydroxide using phenol red indicator. Acetyl esters were determined by treating the marc with 0.1N sodium hydroxide and magnesium sulfate and distilled. The distillate was titrated with 0.05N sodium hydroxide to a phenol red end point (McCready, 1970). Uronic acid content was determined by the method of Gee et al. (1958). Moisture and ash were determined according to the AOAC (1960). The method of Owens et al. (1952) was used for viscosity determination. Since the values of relative viscosity in Kitchener and Alphonso were less than those used in the graph given by Owens et al. (1952), intrinsic viscosity was calculated according to the formula from Doesburg (1965). Weight-average molecular weight was determined from intrinsic viscosity data according to the equation $N = 1.4 \times 10^{-6} M^{1.34}$, where N is the intrinsic viscosity and M is the molecular weight. Results presented are the average of at least three separate determinations that agree well with each other.

RESULTS & DISCUSSION

TABLE 1 shows that there is a variation in pectin content in the three cultivars. The marc obtained from Kitchener was highest but after sieving gave the lowest yield which could be due to the fibres in this cultivar. The color of the marc varied from grayish to white. Alphonso was low in ash (0.017%) compared to the other two cultivars. The carboxyl group influences the viscosity of pectin solution depending upon degree of esterification. Solutions of fully esterified pectin do not change appreciably in viscosity with change in pH, but when they contain pectins of low degrees of esterification, the viscosity becomes markedly pH dependent (Doesburg, 1965). The total carboxyl groups obtained from Abu Samaka were higher than those obtained from Alphonso or Kitchener. The acetyl content varied from 0.412 in Abu Samaka to 0.565 in Alphonso. Pectin acetyls are important in the gelatinization of pectins.

Generally a pectin with a degree of esterification of 70% will form a jelly rapidly or at a higher temperature than one with 50–70%. This investigation

Table 1–Chemical analysis of marc prepared from fresh mango pulp

	Pectin content		Marc[a]											
Cultivar	Total "fresh basis" (%)	Soluble "fresh basis" (%)	Weight before sieving (g)	Weight after sieving (g)	Color	Moisture (%)	Ash (%)	Equivalent weight	Free carboxyl group (mg/g)	Total carboxyl group (mg/g)	Esterified carboxyl group (mg/g)	Acetyl (%)	Degree of esterification (%)	AU (%)
Alphonso	0.265	0.073	2.75	2.45	Whitish grey	6.30	0.017	1505	0.665	1.591	0.926	0.565	58.2	28.0
Kitchener	0.298	0.080	3.20	1.30	Dull white	5.57	0.908	1805	0.555	1.876	1.321	0.556	70.3	33.0
Abu Samaka	0.423	0.220	2.60	2.30	White	5.40	0.60	1858	0.539	2.193	1.654	0.412	75.5	38.5

[a] Weight of marc was obtained from 200g of fresh pulp

Table 2—Relative, specific intrinsic viscosity and molecular weights of pectin extracted from mango marc

Cultivar	Conc (g/100)	Relative viscosity	Specific viscosity	Intrinsic viscosity (1)[a]	Intrinsic viscosity (2)[a]	Molecular weight
Kitchener	0.1	1.089	0.089	0.89	–	2.442×10^4
Alphonso	0.1	1.060	0.060	0.60	–	1.595×10^4
Abu Samaka	0.1	1.122	0.122	1.22	1.20	2.710×10^4

[a] (1) Obtained by calculation; (2) Obtained from the graph after Owens et al. (1952)

showed that degree of esterification of pectin from Alphonso was low (58%) while that from Kitchener or Abu Samaka was 70 and 75%, respectively. The anhydrounic acid (AUA) value was variable in the three cultivars (33.0, 28.0 and 38.5%) Maqbool and Rahman (1968) reported that the marc of Indian variety of mango Langra, contains 71.4% esterification and 24.6% AUA.

Table 2 shows the relative, specific and intrinsic viscosity and weight-average molecular weight of pectin. Intrinsic viscosity was 0.60, 0.89 and 1.22 for Alphonso, Kitchener and Abu Samaka, respectively. The result for intrinsic viscosity from Abu Samaka, calculated according to the formula from Doesburg (1965), was similar to that calculated from the graph as described by Owens et al. (1952). This supports the values for the other two varieties which are obtained by calculation.

The weight-average molecular weight of pectin obtained from Alphonso, Kitchener and Abu Samaka was 1.6×10^4, 2.1×16^4 and 2.7×10^4, respectively. This indicates that the quality of pectin (high viscosity and molecular weight) may affect the viscosity of mango nectar in addition to the quantity of pectin as reported by Saeed (1974) and Saeed and Khattab (1974).

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A Research Note

VOLATILE COMPONENTS OF ROASTED MACADAMIA NUTS

INTRODUCTION

IN RECENT YEARS, spurred largely by the development of the interfaced gas chromatograph-mass spectrometer (GC-MS), numerous studies of complex flavor component systems have been reported. Due perhaps to their wide flavor appeal, roasted nuts have been represented by studies concerning the volatile components of filberts (Kinlin et al., 1972; Sheldon et al., 1972), pecans (Wang and Odell, 1972), and especially peanuts (Walradt et al., 1971; Johnson et al., 1971a, b). These and many additional studies of similar flavor systems have begun to provide a great quantity of reference data which, used in conjunction with the sophisticated analytical techniques now generally available, greatly facilitate the analysis of flavor component systems of such a complexity as to be virtually intractable a short time ago.

The roasted kernels of macadamia nuts (*Macadamia integrifolia*) have been considered by many consumers to posses the finest quality of flavor among the confectionary nut products. In the State of Hawaii, production of macadamia nuts increased from six million pounds in 1963 to 14.2 million pounds in 1972 (Hawaii Crop and Livestock Reporting Service, 1973). The growing importance of this agricultural commodity has prompted several studies on factors affecting its quality (Cavaletto and Yamamoto, 1970). Presented here is a compilation of the major volatile components of the roasted macadamia nuts, identified or tentatively identified on the basis of their GC retention indices and mass spectra.

EXPERIMENTAL

MACADAMIA NUTS were dry roasted in a stainless steel rotating drum with hot air at 177°C for 20 min. Batches between 500–1,000g were dry ground in a Waring Blendor and placed into a round bottom flask connected to a vacuum train. Two cold traps using liquid N_2 as coolant were used. The volatiles collected for analysis were those in the first cold trap. A pressure of 10^{-1} to 10^{-2} mm Hg was maintained for 24 hr. A heating mantel was used to gradually raise the temperature of the ground nuts from room temperature to 75°C during the collection of the volatiles. A milky aqueous suspension of 1–5 ml was obtained in this manner for each of several batches of nuts. The volatile compounds were analyzed by one of the two following procedures.

Procedure A–Essence extract

To the milky suspension was added 25–50 ml water. The pH of this solution was adjusted to ca 1 by the addition of conc HCl. The resulting acid solution was extracted (3 × 25 ml) with redistilled CH_2Cl_2. The CH_2Cl_2 extracts were combined and concentrated using a rotary evaporator to about 1 ml and subsequently by a stream of dry N_2 to about 15 µl. Analysis of this concentrate resulted in the identification of the neutral compounds indicated in Table 1. The acid solution remaining after this initial extraction was adjusted to pH 8 by the addition of powdered sodium bicarbonate and again extracted with CH_2Cl_2 (3 × 25 ml). The organic phase was concentrated as indicated above and the analysis of this concentrate resulted in the identification of the nitrogen-containing compounds indicated in Table 1. The essence concentrate had a wholesome and intense odor of roasted macadamia nut and the chromatogram produced was similar to the superimposed sum of the chromatograms of the basic and neutral fractions produced as described above.

Procedure B–Headspace analysis

The cold trap containing the milky suspension was removed from the vacuum train and fitted, after the addition of 1–2g anhyd Na_2SO_4, with a ground joint to which was attached a Teflon stopcock. The trap was again placed into liquid N_2 and briefly evacuated through the stopcock by a vacuum pump. The stopcock was then closed, fitted with a silicone rubber septum, and reopened. The cold trap was then removed from the coolant, allowed to warm to room temperature, and finally heated in a water bath to 65°C. Gas samples of 1–3 ml were taken from the trap by means of a gas-tight syringe. Analyses of these headspace samples resulted in the identification of the highly volatile compounds indicated in Table 1.

Gas chromatography

An 18 ft × 1/8 in. stainless steel column packed with 3% Carbowax 20M on acid washed, DMCS treated 80–100 mesh Chromosorb G was used in a Varian Aerograph Series 1800 dual column gas chromatograph fitted with flame ionization detectors. Retention indices (I_E values) relative to the homologous series of ethyl esters of saturated fatty acids were determined based on van den Dool and Kratz (1963). For the essence extracts, a carrier gas (N_2) flow rate of 14 ml/min and an oven temperature programmed from 70–190°C at 1°C/min were used. For the headspace analysis, a carrier gas flow rate of 18 ml/min and an isothermal oven temperature of 60°C were used. In both cases the injection port temperature was 200°C and the detector temperature was 245°C.

Gas chromatography–Mass spectrometry

The column and operating conditions employed for the gas chromatograph in GC-MS analysis were similar to those described above. The instrument used to obtained the mass spectra was a Finnegan Series 3000 GC Peak Identifier interfaced to a Varian Aerograph Series 1400 gas chromatograph. The compounds were identified by comparison of the GC retention indices and mass spectra with reference data from the literature and tables of compiled spectra (Table 1). Approximately half of the compounds were further compared with samples of authentic compounds. In every instance the GC retention and mass spectral data agreed.

RESULTS & DISCUSSION

THE BASIC PORTION of the volatiles consists of a number of pyrazines essentially similar to those found in a great variety of heat-treated foods. Numerous studies indicate that the pyrazines arise mainly from the heat-initiated interaction of amino acids and sugars. (For leading references see the review by Maga and Sizer, 1973). The synthetic work and spectra presented by Bondarovich et al. (1967) are especially helpful in identifying the simple alkylpyrazines.

The neutral and headspace compounds consist largely of alcohols, aldehydes and ketones. It has been suggested that these latter carbonyl compounds are probably derived by means of autoxidation from unsaturated fats (Brown et al., 1973 and ref. therein). The identification of these compounds was greatly facilitated by the ever-increasing body of reference data. Particularly useful were the large compilations of Kinlin et al. (1972) and of Walradt et al. (1971).

It is readily apparent that there are many similarities among the lists of volatile components of the several roasted nuts. Indeed, the similarities are greater than the differences. Yet the flavor of each is somewhat different. Apparently the proportions of the components and/or compounds present in lesser quantities may play an important role *in determining* overall flavor. Methyl sulfide is a major component among the highly volatile compounds found in macadamias, yet

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Table 1—Compounds identified from roasted macadamia nuts

Compound	Retention index	Size of peak[a]	Present in peanuts[b]	Present in filberts[c]	Present in pecans[d]
Headspace					
n-hexane	0.3	S		X	
n-heptane	0.5	M		X	
methyl sulfide	0.6	L			
n-octane	0.8	M		X	
2-methylpropanal	1.0	L	X	X	
methylfuran	1.9	S		X	
2-methylbutanal	2.4	L	X	X	
3-methylbutanal	2.5	L		X	
benzene[e]	2.8	S	X	X	
toluene[e]	4.0	S	X	X	
methyl disulfide	4.4	S	X	X	
Essence extract—Neutral					
2,3-pentadione	4.1	S	X	X	X
n-hexanal	4.5	M	X	X	X
p-xylene	5.0	S	X	X	
n-heptanal	5.5	M	X	X	X
2-heptanone[e]	5.5	S	X	X	
n-pentanol	6.0	S	X	X	X
2-pentylfuran[e]	6.0	S	X	X	
p-cymene[e]	6.3	S	X	X	
2-methyltetrahydrofuran-3-one[e]	6.4	S		X	
n-octanal	6.6	M	X	X	X
n-hexanol	7.0	M	X	X	X
n-nonanal	7.7	L	X	X	
n-heptanol	8.1	M	X	X	X
2-furfural	8.2	S	X	X	X
3-methylthiopropion-aldehyde	8.2	S	X	X	
benzaldehyde	9.0	S	X	X	
n-octanol	9.1	M		X	X
3,5,5-trimethyl-2-cyclohexen-1-one[e]	9.6	S		X	
phenylacetaldehyde	10.1	L	X	X	
1-phenyl-2-propanone	10.9	M	X	X	
2-phenylethanol	12.7	S	X	X	
2-phenyl-2-butanal	13.0	S		X	
phenol	13.3	M		X	
Essence extract—Basic					
2-methylpyrazine	6.4	S	X	X	X
2,5-dimethylpyrazine	7.0	L	X	X	X
2,3-dimethylpyrazine	7.2	S	X	X	X
2-ethyl-5-methyl-pyrazine	7.7	S	X	X	X
2,3,5-trimethyl-pyrazine	7.8	M	X	X	X
2-ethyl-3,6-dimethyl-pyrazine	8.2	L	X	X	X
2,5-diethyl-3-methyl-pyrazine[e]	8.7	S		X	

[a] Estimate of peak size relative to size of other peaks in the same group. The symbols L, M, S indicate large, medium and small, respectively.
[b] Walradt et al., 1971
[c] Kinlin et al., 1972
[d] Wang and Odell, 1972
[e] Tentative identification

the compound has not been reported as among those present in peanuts, filberts or pecans. It has, however, been reported among the volatiles found in roasted cocoa beans (van Praag et al., 1968). Since it has been qualitatively observed that longer roasting periods yeild macadamias with a more "peanut-like" flavor (Cavaletto, 1973), it could be that methyl sulfide plays a role in the characteristic macadamia flavor. Being highly volatile, it would be driven off by prolonged roasting. Additional studies are being pursued regarding this possibility.

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A Research Note

PREVENTION OF SKIN SHRINKAGE IN CUT-UP HOT-PACKED BROILERS

INTRODUCTION

IN RECENT YEARS, the increasing demand by consumers for more convenience food items has stimulated the growth of marketing cut-up broilers. According to the most recent survey made by the National Broiler Council (National Broiler Council, 1971) in 1970, 74% of the broiler processors were engaged in producing cut-up chicken, and about 25.3% of the processors' volume sold in the United States was in various cut-up forms. The vast majority of processors also increased their sales of cut-up birds. The recent trend among poultry processors has been toward performing the cutting and packaging at the processing plant.

Systems which eliminate ice and water chilling in poultry processing have been reported to improve the quality of packaged poultry (Anonymous, 1970). The "hot packaging" of broilers (without ice-slush chilling), as reported by Chen (1972), yields a better product with better microbiological quality, has less drip formation and organoleptically no significant difference was found when compared with those of ice-slush chilled samples. The banning of immersion chilling of poultry in the European market on Jan. 1, 1977, as per the resolution by the European Economic Community (EEC) in 1971, suggests the need for research for an alternative method which can eliminate the immersion chilling step in the process. Hot packaging of birds followed by air chilling or refrigeration could be a solution to this problem.

With the change in processing steps, some problems have arisen. It was observed in our laboratory that the cutting of hot packed broilers yielded an unappealing product due to the severe skin shrinkage of the parts. This study was to observe the skin shrinkage phenomenon of cut-up warm carcasses and to find a solution to it.

MATERIALS & METHODS

BROILERS fed a commercial-type diet were slaughtered at 9 wk of age at the Mississippi State University Poultry Processing Plant. The birds were killed by the commercially used semi-kosher cut, scalded at approximately 54.4°C and picked in a batch-type picker. Carcasses were eviscerated using commercial techniques and rinsed inside and out with a spray of cold water. The washed carcasses were immediately packed in Cryovac bags as the "hot" sample. Control carcasses consisted of broilers treated in like manner except that they were chilled in ice-slush for approximately 90 min.

The subcutaneous layer temperatures of the ventral skin area were measured from each carcass with thermocouples and were recorded with a Speedomax G recorder. The carcasses were cut along the transverse plane at the ventral center and the drumsticks were separated. The cut-up parts were placed on a plastic tray and covered with Saran film to prevent the loss of moisture. The wrapped cut-up parts were immediately stored at 2–4°C in a walk-in refrigerator. Distances in mm between the edge of skin and the edge of muscle of the cut-up plane were measured and averaged. Measurements were made at 1-hr intervals for 6 hr after the 0-, 0.5- and 1.0-hr readings. Experiments were repeated 10 times and data pooled. Several observations were made for 21 days.

The effects of subcutaneous layer temperatures on the skin shrinkage of cut-up parts were

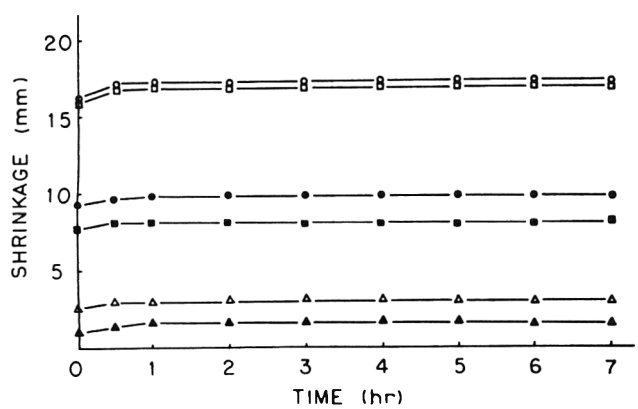

Fig. 1—Shrinkage of various skin regions of cut-up broilers stored at 2–4°C. Symbol: ○, ventral skin, hot packed; □, knee skin, hot packed; △, dorsal skin, hot packed; ●, ventral skin, ice-chilled; ■, knee skin, ice-chilled; ▲, dorsal skin, ice-chilled.

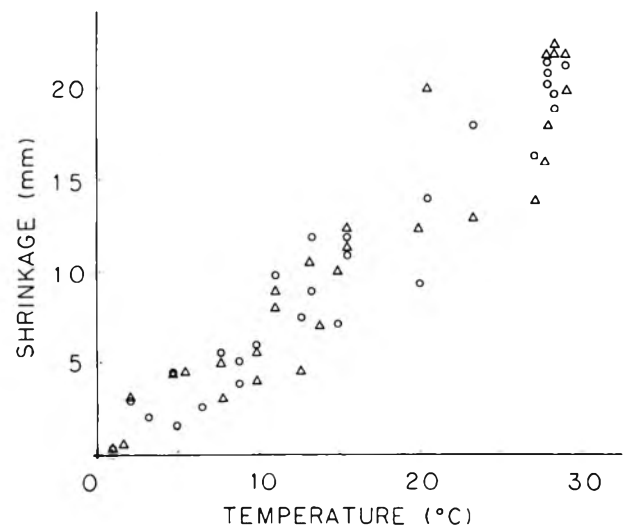

Fig. 2—Effect of subcutaneous layer temperatures on the skin shrinkage of cut-broilers. Symbols: ○, ventral skin; △, knee skin.

investigated. The warm carcasses were packed individually in Cryovac bags and placed in a blast-type freezer. Birds were removed at 5 min intervals and the subcutaneous layer temperatures of the ventral skin were measured with thermocouples. The carcasses were then cut-up as described previously. Skrinkages of skin in mm on the ventral and the knee regions were measured and averaged. Thirty observations for various subcutaneous layer temperatures were made.

RESULTS & DISCUSSION

SKIN SHRINKAGE of the cut-up broiler parts was observed from both the "hot-packed" and the ice-slush chilled carcasses. Three areas were observed with the ventral and knee regions having the highest degree of shrinkage while the dorsal skin had the lowest (Fig. 1). The severe skin shrinkage of the cut-up hot-packed broiler parts resulted in an unappealing product. Ice-sluch chilling of the carcass did not prevent the skin shrinkage after cutting. However, the skin of ice-slush chilled carcasses shrank much less than that of hot-packed carcasses. The chilled carcasses used had an average internal temperature of 11.1°C with 14.4 ± 2.6°C subcutaneous layer temperature before cutting. This internal temperature of 11.1°C was higher than those of 3.3–4.4°C in the commercial operations.

Skin shrinkage took place almost instantaneously after cutting. For both the hot and the ice-chilled samples, the degree of shrinkage after cutting remained essentially the same throughout the entire refrigerated period. As Figure 1 shows, only a very slight increase in shrinkage was observed in the first 30 min of refrigerated storage. Several observations were made for 21 days, and no increase in shrinkage was recorded. Improper packaging of the cut-up parts led to surface dehydration and increased the degree of shrinkage.

Lower subcutaneous layer temperatures, achieved by placing the warm birds in a blast freezer for a short period, resulted in lower degrees of skin shrinkage after cutting (Fig. 2). The correlation coefficients between the subcutaneous layer temperatures and the skin shrinkage after cutting for ventral and knee area skin were calculated to be 0.95 and 0.84, respectively. The shrinkage of skin after cutting could be completely prevented by lowering the subcutaneous layer temperature to 1.1°C. Liquid nitrogen spraying, Freon tunnel chilling or other similar processes can also be used to lower the surface temperature of the carcasses.

According to USDA regulations, broiler carcasses must be chilled to below 4.4°C to adequately protect the product. If the subcutaneous layer temperature of the carcasses were rapidly lowered, the growth of microorganisms on the surface would be arrested by the low temperature. The internal temperature of the cut-up part would be gradually equalized and lowered by refrigerated storage.

Over-chilling of the carcasses would result in a frozen surface. This frozen crust formation is not desired for the hand cutting process. With 1–3°C subcutaneous layer temperature, a soft, cheese-like meat texture would be obtained during cutting. This semi-firm texture would facilitate handling in the cutting process.

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A Research Note
MATHEMATICAL ANALYSIS OF SOLUBILIZATION KINETICS AND DIFFUSION IN FOODS

INTRODUCTION

SOLUBILIZATION, the reaction-induced conversion of insoluble precursors into readily recoverable or removable soluble products, is widely employed in the food industry. Solubilization usually occurs as a result of hydrolysis within a water-swollen solid matrix. To recover the solubles produced they must be leached or extracted from the solid in which they are formed into liquid which surrounds the solid. In terms of product recovery, solubilization and leaching are intimately linked.

Examples of solubilization include: the conversion of hemicellulose into oligosaccharides during the high temperature extraction of coffee; the conversion of collagen into gelatin; the release of pectin from protopectin; the conversion of grain-bound starches into sugars during the preparation of brewing worts; the release of tannins from tannin complexes during the extraction of tea; and the hydrolysis of linamarin in cassava followed by the subsequent extraction of the HCN released.

Potential uses of solubilization include: acid and the enzyme catalyzed saccharification of cellulose, Reese et al., 1972; conversion of sclero-proteins into usable proteins; recovery of polypeptides from murein; conversion of high-molecular-weight cell-bound proteins into smaller protein fragments which can diffuse through cell walls and be recovered by leaching, detoxification of toxin containing foods, and chemical pretreatment of food to augment the availability of nutrients through subsequent natural solubilization processes in the digestive tract.

In analyzing solubilization processes it is important to separate kinetic effects and diffusional effects. The solubles created are often subject to secondary degradation reactions. To minimize such degradation and to adequately account for effects produced by changes in particle size it is important to know both the rate constant for the solubilization reaction itself and the diffusivity of the solubles in the solid substrate. The objective of this paper is to develop mathematical techniques for determining such values from experimental data.

MATHEMATICAL ANALYSIS

WE WILL CONSIDER the analysis of a first-order or pseudo-first-order solubilization reaction occurring within a solid which, at least on a skeletal basis, remains structurally intact. This analysis is not applicable when the solubilization reaction takes place only at the outer surface of the solid involved. Since many solubilization reactions are hydrolyses and water is present in great excess, the assumption of a pseudo-first-order reaction is often valid.

Solubilization alone

If insoluble solids immersed in liquid undergo a first-order or pseudo-first-order solubilization reaction and there is no diffusional resistance to the release of the solubles created, C the solubles concentration per unit volume of liquid will be given by the expression:

$$\frac{C}{C_\infty} = 1 - \exp[-k\theta] \tag{1}$$

where θ is the reaction time, k is the reaction rate constant and C_∞ is the solubles concentration that would be obtained after an infinite reaction time provided no solubles breakdown occurs. C_∞ is related by the following material balance to F the solids volume, L the liquid volume, and S the mass of solubles generatable per unit volume of solids:

$$C_\infty = \frac{FS}{L + F/m} = \frac{mS}{\alpha + 1} \tag{2}$$

where $\alpha = Lm/F$, and m is the equilibrium ratio of the concentration of the solubles in the free liquid to the concentration of solubles in the solid. When the solubles in the solid are not selectively adsorbed, m is the reciprocal of the volume fraction of the solid which is occupied by imbibed solution. It is assumed that m and the volumes L and F remain constant.

Eq (1) can be rearranged to yield:

$$\log\left[\frac{C_\infty - C}{C_\infty}\right] = \frac{k\theta}{2.303} \tag{3}$$

Therefore, if C_∞ is known from experiments carried out at long reaction times, one can determine k from the slope of a plot of $\log[(C_\infty - C)/C_\infty]$ vs. θ.

In analyzing solubilization test data it is usually observed that such plots are curved for small and intermediate values of θ, but that for moderately large values of θ, the plots are straight with a negative slope. It is very frequently assumed that initial curved portions of these semi-log plots deviate from Eq (3) because of transient conditions which occur during test start-ups and that these transients are no longer significant at large values of θ. As a consequence it is further assumed that k can be correctly determined from the slope of the straight line portion of the semi-log plot. Unfortunately, these assumptions are often invalid.

Diffusion alone

Purely diffusive release of available solubles will also lead to a plot of $\log[(C_\infty - C)/C_\infty]$ vs. time which has a negative slope, is curved for small values of time and is straight for large values of time. If solids which contain available solubles or instantly solubilized material are immersed in well-stirred liquid, the diffusive release of the solubles is governed by an equation of the type

$$\frac{(C_\infty - C)}{C_\infty} = \sum_{n=1}^{\infty} B_n \exp[-q_n^2\, Dt/a^2] \tag{4}$$

Where B_n and q_n are terms whose values depend on the geometry of the particles making up the solid and the quantity α which was previously defined.

D is the diffusivity of the solute in the solid, a is a characteristic dimension for the solid, and t is the immersion time. Values of a, B_n and defining equations for q_n for different geometries are listed in Table 1. Eq (4) and the values in Table 1 were obtained by rearranging equations which are available in Crank, 1956.

The diffusional release of solubles from sheet-like material such as leaves is reasonably well governed by the infinite slab form of Eq (4); that from stems, stalks and circular rods by the infinite cylinder form; and that from seeds and ground granules by the sphere form. The

relationship of C_∞ to the solubles content of the solids is given by Eq (2) where we now take S to be the mass of immediately available solubles per unit volume of solids.

For values of Dt/a^2 larger than 0.1 the first term of the series can usually be used by itself with a relative error of less than 2%. Thus for $Dt/a^2 > 0.1$, the plot of log $[(C_\infty - C)/C_\infty]$ vs. t will be a straight line. The slope of this line, $-Dq_1^2/2.303\ a^2$ as determined from tests measuring the release of previously absorbed solubles can be used to calculate the diffusivity D if a and q_1 are known. The value of q_1 is solely a function of α which along with a, can be determined from measurements made prior to a diffusion test. Unfortunately $(C_\infty - C)/C_\infty$ will range from about 0.4 for slabs to 0.1 for spheres at low α when $Dt/a^2 = 0.1$. Therefore C_∞ and C must be measured quite accurately if the slope of the semi-log plot is to be used to determine D.

Solubilization and diffusion combined

If solubilization occurs in a solid which offers diffusive resistance to the transfer of solubles, the differential increment in concentration in the surrounding fluid at time t due to the incremental solubilization occurring at time θ is given by

$$dC = dC_\theta \left\{ 1 - \sum_{n=1}^{\infty} B_n \exp \left[\frac{-q_n^2 D(t-\theta)}{a^2} \right] \right\} \quad (5)$$

where dC_θ is the change in concentration that would have been produced during time interval $d\theta$ if there had been no diffusive resistance. Eq (5) is obtained by rearranging Eq (4) and substituting dC_θ for C_∞. From Eq (1),

$$dC_\theta = \frac{C_\infty}{k} \exp\left[-k\theta\right] d\theta \quad (6)$$

Substituting for dC_θ in Eq (5), integrating from $\theta = 0$ to $\theta = t$ and rearranging there is obtained:

$$\frac{C_\infty - C}{C_\infty} = e^{-kt} \left\{ 1 - \sum_{n=1}^{\infty} \left[\frac{B_n}{1 - \frac{q_n^2 D}{a^2 k}} \right] \right\} + \sum_{n=1}^{\infty} \left[\frac{B_n}{1 - \frac{q_n^2 D}{a^2 k}} \right] \exp \left[-\frac{q_n^2 Dt}{a^2} \right] \quad (7)$$

For large t this becomes

$$\frac{C_\infty - C}{C_\infty} = e^{-kt} + \left[\frac{B_1}{1 - \frac{q_1^2 D}{a^2 k}} \right] \left\{ \exp \left[\frac{-q_1^2 Dt}{a^2} \right] - \exp\left[-kt\right] \right\} \quad (8)$$

Table 1—Parameters for diffusional release

Geometry	B_n	a	q_n The nth non-zero positive roots of
Infinite slab	$\frac{2\alpha(1+\alpha)}{1+\alpha+q_n^2\alpha^2}$	half thickness	$\tan q_n = -\alpha q_n$
Infinite cylinder	$\frac{4\alpha(1+\alpha)}{4+4\alpha+q_n^2\alpha^2}$	radius	$-\alpha q_n J_0(q_n) = 2J_1(q_n)$*
Sphere	$\frac{6\alpha(1+\alpha)}{9+9\alpha+q_n^2\alpha^2}$	radius	$\tan q_n = \frac{3q_n}{3+\alpha q_n^2}$

* J_0 and J_1 are Bessel functions of order zero and order 1, respectively.

Eq (8) reduces to Eq (1) when $q_1^2 D/a^2 \gg k$ and it reduces to the first term of Eq (4) when $k \gg q_1^2 D/a^2$. Unless $q_1^2 D/a^2 k > 3$ one cannot accurately determine k from the slope of log $(C_\infty - C)/C_\infty$ vs. t.

Log $(C_\infty - C)/C_\infty$ is plotted vs. kt, for $\alpha = 4$ and various $q_1^2 D/a^2 k$ ratios in Figure 1. when $q_1^2 D/a^2 k > 3$ Eq (8) reduces to

$$\frac{C_\infty - C}{C_\infty} = e^{-kt} \left\{ 1 + \left[\frac{B_1}{\frac{q_1^2 D}{a^2 k} - 1} \right] \right\} \quad (9)$$

with an error of 2% or less when $e^{-kt} \leq 0.4$. The slope for a plot or log $[(C_\infty - C)/C_\infty]$ vs. t at large t will be $-k/2.303$, as in the case of Eq (1). Further, once k has been determined, Eq (9) can be used to solve for D, provided a^2 is not too small.

RESULTS & DISCUSSION

Example

As an example let us consider the solubilization of hemicellulose in spent coffee grounds. In tests carried out at 150°C using 1.5 volumes of water per volume of wet grounds (i.e., L/F = 1.5) an aqueous solution containing 4.45% soluble solids is obtained at 20 minutes reaction time, 5.65% at 40 min and 6.0% after 200 min and longer. Wet spent coffee grounds contain about 68% water by volume. Therefore, m = 1/0.68 = 1.47; and α = Lm/F = 1.5 × 1.47 = 2.20. By iteration (two steps) the solution of the q_1 equation for spheres in Table 1 is $q_1 = 3.48$; and $B_1 = 0.483$. The grounds have a diameter of 0.1 cm, so a = 0.05 cm.

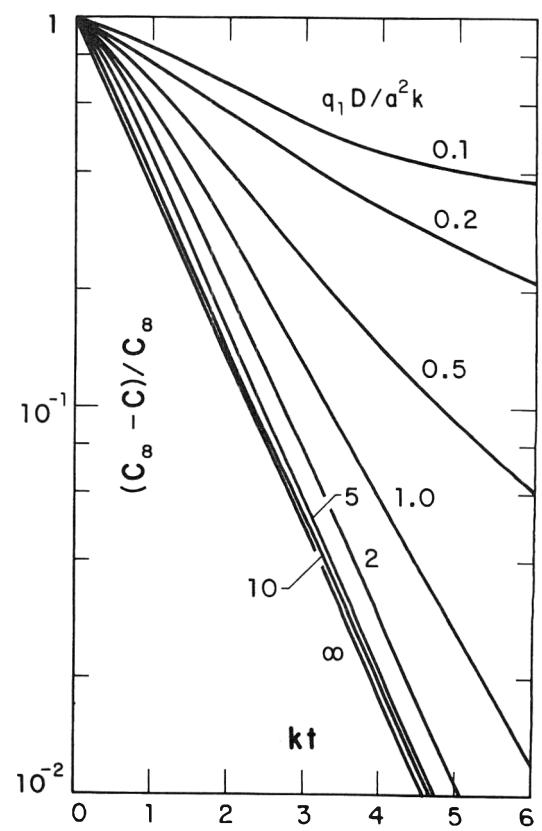

Fig. 1—Released solute concentration as a function of solubilization reaction time at various levels of diffusional resistance (for spherical particles).

Since $(C_\infty - C)/C_\infty = 1.55/6.0 = 0.258 < 0.4$ at 20 min, assuming $q_1^2 D/a^2 k$ is large enough, we can determine D from the slope of the semi-log plot of $(C_\infty - C)/C_\infty$ vs. t, i.e.,

$$k = \frac{2.303}{t_2 - t_1} \log \left[\frac{C_\infty - C_1}{C_\infty - C_2}\right] = \frac{2.303}{(40 - 20)60 \text{ sec}} \log \left[\frac{6.0 - 4.45}{6.0 - 5.65}\right]$$

or

$$k = 1.24 \times 10^{-3} \text{ sec}^{-1}$$

Eq 9 rearranged to solve for D yields

$$D = \frac{a^2 k}{q_1^2} \left[1 + \frac{B_1}{\dfrac{C_\infty - C_1}{C_\infty(e^{-kt})} - 1}\right] \qquad (10)$$

$$D = \frac{25 \times 10^{-4} \text{ cm}^2 \times 1.24 \times 10^{-3} \text{ sec}^{-1}}{(3.48)^2} \times \left[1 + \frac{0.483}{\dfrac{6.0 - 4.45}{6.0(e^{-1.24 \times 1.2})} - 1}\right]$$

$$D = 1.1 \times 10^{-6} \text{ cm}^2/\text{sec}$$

when $t = 20 \times 60 = 1200$ sec and the corresponding concentration is substituted. The same answer is obtained at $t = 40 \times 60 = 2400$ sec. Because of its sensitivity to small changes in C, Eq (10) does not yield very accurate values of D, but the value obtained in the present case is about right for coffee grounds. Using this value, $q_1^2 D/a^2 k = (3.48)^2 \times 1.1 \times 10^{-6}/(25 \times 10^{-4} \times 1.24 \times 10^{-3}) = 4.29 > 3$; so k was validly determined from the slope of the semi-log plot. The value of k is about right for coffee grounds at 150°C. At higher temperatures a smaller particle size would probably have to be used for k to be accurately determined.

Unfortunately, one usually doesn't know k or D ahead of time. In fact, values of D obtained before solubilization may not be useful since, as in the case of coffee grounds, D may increase by a factor of 4 or 5 during solubilization–particularly when cell wall constituents are being solubilized. When prior estimates of k and D are not available to assist one in choosing the best value of a for finding refined values of k and D, it is desirable to make a as small as is feasible. Generally, if a is about 0.01 cm there should be no trouble measuring k, providing k is not extremely large (in which case one would not care what k was), but with such a small a, there may be trouble due to pressure drop and imperfect filtration when withdrawing samples during the solubilization test. Once k has been determined by tests using a small particle size, its value can be checked and D can be determined through the use of Eq (9) by tests carried out using larger sized particles.

From a practical point of view, finer grinding will yield only minor reductions in processing time if $q_1^2 D/a^2 k > 2$. If filtration and pressure drop difficulties can be avoided, substantial reductions in processing time and possibly marked improvements in product quality can be obtained by finer grinding when $q_1^2 D/a^2 k < 0.5$.

If the tests are carried out with at least two widely different solids-to-liquid ratios (i.e., $\alpha \approx 1.0$ and $\alpha \geqslant 4.0$) one will be able to distinguish whether any diffusional effects influence the slope of log $[(C_\infty - C)/C_\infty]$ vs. t. In the absence of a diffusional effect, the slope will not change when the solids-to-liquid ratio is changed, but if diffusional effects persist there will be a detectable change in slope.

Multiple substrates and breakdown reactions

Changes in particle size and solids-to-liquid ratio will also prove useful in detecting a variety of kinetics effects which may be important. If there is a multiplicity of insoluble substrates each of which is solubilized according to a different rate constant, the plot of log $[(C_\infty - C)/C_\infty]$ vs. t will be curved even in the absence of any diffusive effects. The initial slope in the absence of diffusional resistance will be that corresponding to the weighted average of the reaction rate constants for all the substrates and the final slope will be that corresponding to the lowest reaction rate constant. If this slope pattern persists when particle size is reduced and the solids-to-liquid ratio is changed one can be reasonably sure one is dealing with multiple substrates and that rapid complete solubles release cannot be obtained merely through the use of finer grinding.

Difficulty will be encountered in determining C_∞ when solubles breakdown reactions take place. By using very small values of a the plot of log $[(C_\infty - C)/C_\infty]$ vs. t should be straight even at relatively small values of t. Therefore, if the plot is not straight (and if multiple substrates are not present)–and particularly if there is evidence of a reduction in C at large values of t–one should determine that value of C_∞ which produces as reasonably straight a line as possible at small to intermediate values of t. Since many solubles breakdown reactions are higher order reactions, their speed will be minimized and yields will be improved by running at as high a dilution (i.e., a high liquid/solids ratio) as is feasible. If C can be measured accurately at high dilution the determination of C_∞ by trial and error or regression may be greatly facilitated. Determination of C_∞ will not only provide a measure of the maximum attainable yield, but will provide a reference against which the solubles breakdown losses can be determined.

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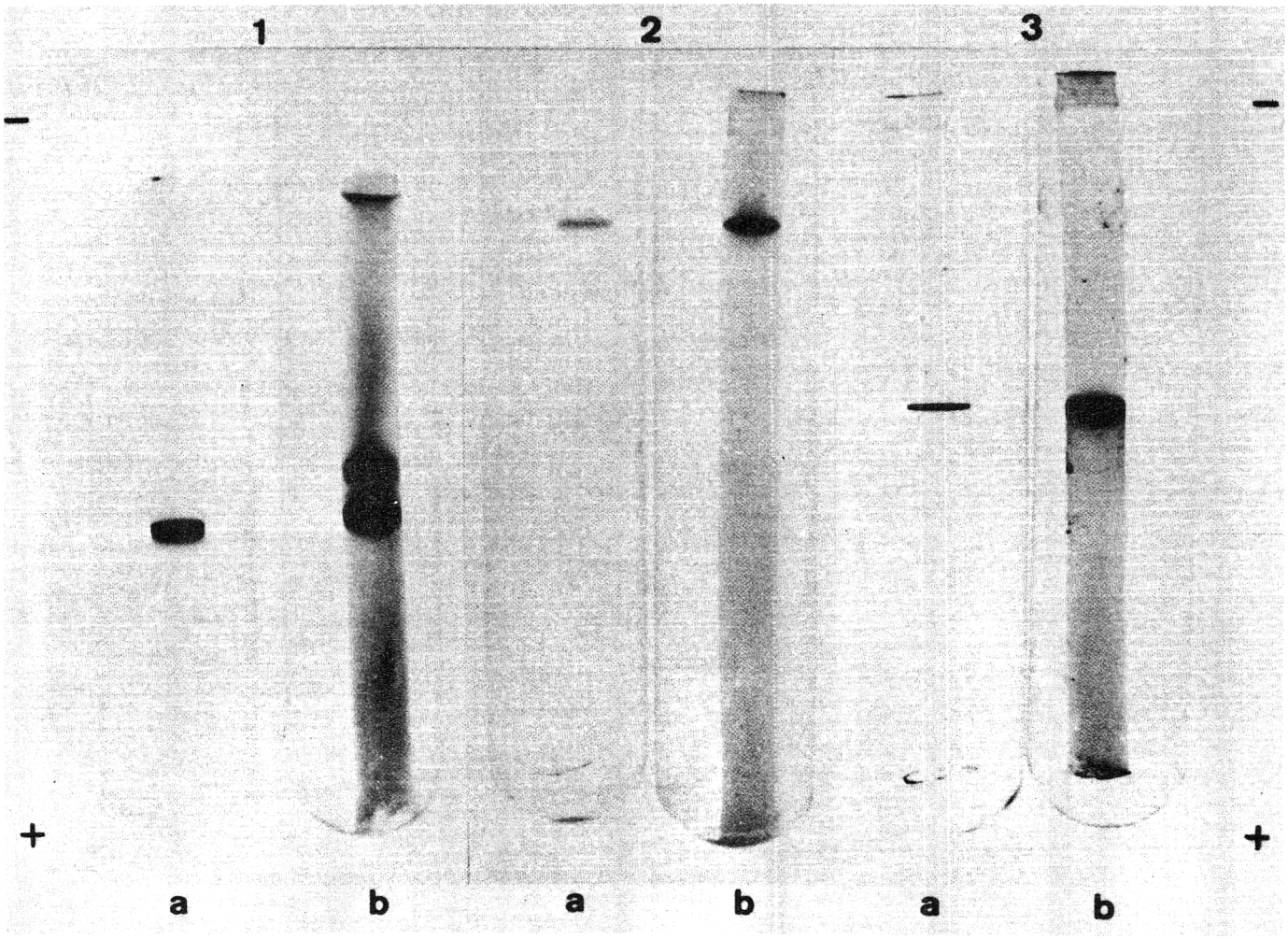
Erratum Notice

● *J. Food Sci.* 39(4): 786–791, P.G. Pifferi and R. Cultrera: "Enzymatic degradation of anthocyanins: The role of sweet cherry polyphenol oxidase." On page 788, Figure 3, delete the photograph and insert the correct photograph printed herewith; the caption is correct as printed.



Naturally Occurring Toxicants in Foods

A Scientific Status Summary by the Institute of Food Technologists' Expert Panel on Food Safety and Nutrition

Any comprehensive consideration of the toxicology of the natural chemical components of foods should take into account the total picture of foods as the most complex part of man's chemical environment. The origins of potentially harmful chemical substances that occur in foods are shown in the accompanying table, which constitutes a skeleton outline of the subject of food toxicology. (Infectious organisms that are important hazards as food contaminants are not included in this outline, because the illnesses they produce are not ordinarily considered to be toxicological processes.)

Many of the chemical substances present in foods because of natural processes are the same as those that enter foods as a result of man's efforts to produce or process foods, or to distribute and prepare them for immediate consumption. For example, a predominant number of the food additives (e.g., vitamins; amino acids; fatty acids and their esters; polysaccharides; inorganic and organic salts of sodium, potassium, calcium, and magnesium; compounds of other essential elements; spices and flavors; and natural coloring materials) are normal constituents of natural food products. Such toxic metals as lead, mercury, cadmium, arsenic, and zinc, which may contaminate foods from utensils or through environmental pollution, are also normally and naturally present in them. In fact, they are unavoidable, since they occur in soil, water, and plants from the natural geochemistry of the earth. Substances generated by chemical changes caused by heat, drying, freezing, pickling, and irradiation are obviously derived from the natural chemical components of the foods subjected to these processing methods.

On a worldwide basis, substances in categories 1 and 2a have produced greater known injury to man than have those in the other categories. Category 7 has also made a substantial, though lesser, contribution to the total incidence of food-borne illness. On the other hand, categories 3 to 6 are not known to have been responsible for adverse effects on human health when such materials have been used in accordance with good agricultural and manufacturing practices.

These comparisons of the extent of the known injury to human health caused by the different groups of

TOXIC CHEMICALS IN FOODS

Natural

1. Normal components of natural food products
2. Natural contaminants of natural food products
   a. Microbiological origin: toxins
   b. Non-microbiological origin: toxicants (e.g., Hg, Se) consumed in feeds by animals used as food sources

Man-made

3. Agricultural chemicals (e.g., pesticides, fertilizers)
4. Food additives
5. Chemicals derived from food packaging materials
6. Chemicals produced in processing of foods (e.g., by heat, ionizing radiation)
7. Inadvertent or accidental contaminants
   a. Food preparation accidents or mistakes
   b. Contamination from food utensils
   c. Environmental pollution
   d. Contamination during storage or transport

agents appear to parallel the comparative susceptibility of the groups to regulatory control. Thus, what man has used intentionally to produce, process, and package foods can be subjected to rigid controls, whereas the natural composition of foods and the natural processes of food contamination are relatively immune to regulatory measures.

Common Foods Contain Nutrients – and Toxicants

The normal components of natural food products constitute more than 99% of the weight of our daily diet. Intentional food additives comprise the bulk of the balance, with pesticide residues and contaminants of both natural and man-made origin contributing only trace amounts, on the order of parts per million. When it is considered that most of the food additives are themselves either dietary supplements or materials derived from or present in natural sources, it becomes clear that only a small fraction of 1% of our diet is *not* derived from natural food products.

Thus, from a toxicological standpoint, the normal and natural constituents of foods contribute by far both the greatest amount and the widest variety of chemical substances consumed by man over his lifetime. In spite of that fact, no single plant used as a source of food has been as well characterized chemically as has the air we breathe and the water we drink, even when these are quite polluted.

For example, the potato, usually thought of as one of man's simpler foods, is a complex chemical aggregate. About 150 distinct chemical substances have been identified in this natural product, among which are the solanine alkaloids, oxalic acid, arsenic, tannins, nitrate, and over a hundred other items of no recognized nutritional significance to man (Talburt and Smith, 1967). Forty-two chemical entities have been found in orange oil (Coleman and Shaw, 1971), including 12 alcohols, 9 aldehydes, 2 esters, 14 hydrocarbons, and 4 ketones. The orange as a whole includes a host of other chemical substances. All vegetables and fruits and other natural food products are similarly complex.

Are Toxicants Always Hazards?

Relatively few of the specific chemicals known to be naturally present in our foods have been evaluated toxicologically. Furthermore, if almost any of these chemical substances were tested in experimental animals by today's standards of safety evaluation, it would be shown to be toxic. This inevitable presence of thousands of toxic substances in natural foods does not imply, however, that a hazard exists.

The *toxicity* of a substance is its intrinsic capacity to produce injury when tested by itself. The *hazard* of a substance is its capacity to produce injury under the circumstances of exposure. Our concern over the safety of natural food products is not, therefore, directly with the intrinsic toxicity of their innumerable chemical components, but rather with the potential hazards of these materials when we eat the foods in which they are present.

In spite of the multitude of toxic substances consumed daily in a normal diet by normal healthy individuals, there is little evident hazard involved. There are three basic interrelated explanations for this:

- **First**, the concentration of each of the toxic substances in any commonly accepted food is so low that a grossly exaggerated consumption of the food, usually over an extended period of time, is required before its toxicity can be translated into a hazard. Such situations have resulted, for example, when cabbage, which contains goitrogenic substances (VanEtten and Wolff, 1973), has constituted an excessively large proportion of the diet for a long time, and following the daily consumption of a half-gallon of tomato juice, which contains lycopene, for several years (Reich et al., 1960). If one's diet contains a reasonable diversity of foods and no extraordinary amount of any specific food, then it is unlikely that any single chemical will be consumed in a toxic amount.

- **Second**, the toxicities of the thousands of different chemicals present in our diet each day are not additive. For example, if a hundredth of the lethal dose of each of a hundred different food components of variable biological actions were combined, the mixture would be innocuous. The human organism can readily tolerate small amounts of many different chemical substances taken simultaneously, even though any one of them might not be tolerated in a somewhat larger amount.

- **Third**, numerous examples of antagonistic interactions among the trace elements have been demonstrated in animals (Underwood, 1973). In these instances, the toxicity of one element is offset by the presence of an adequate amount of another element. For example, the effects of a toxic level of cadmium in the diet are reduced by an accompanying high level of zinc. The adverse effect of manganese, which interferes with the absorption of iron, can be offset by additional iron in the diet. Copper antagonizes the toxic effect of high dietary levels of molybdenum. Evidence of antagonisms between selenium and mercury (Ganther et al., 1972) and between cobalt and iron (Bell, 1973) as dietary factors has been reported. Similarly, iodine inhibits the action of some goitrogens.

The existence and frequency of these antagonisms reinforce the concept of "safety in numbers" as applied to the diet. The wider the variety of food intake, the greater is the number of different chemical substances consumed and the less is the chance that any one chemical will reach a hazardous level in the diet. This principle has been recognized as applying also to food additives and pesticides. The Joint FAO/WHO Expert Committee on Food Additives (FAO/WHO, 1967) stated:

> From the toxicological point of view there is less likelihood of long exposure, or of high or cumulative dose levels being attained if a wide range of substances is available for use. Similar considerations apply to pesticides.

Many Factors Influence the Hazard Potential

Technology has been largely responsible for protecting man against the potential hazards of the natural chemical composition or contamination of his foods. Heating or other

processing methods destroy or remove such toxic components as the cyanogenetic glycosides and some of the goitrogens. Refrigeration, canning, packaging, and other preservation measures suppress contamination by microbial toxins and chemical changes in foods that might generate toxic materials.

Furthermore, the widespread geographical distribution of food products results in the consumption by the population in a given geographical area of foods produced in a variety of other areas (Underwood, 1973). Hazards that might arise from geochemical imbalances or other localized environmental factors—if a population consumed only those foods produced in its own area—are thus largely obviated. The food distribution system that has contributed to this protection has been made possible by modern food production and processing technology, including the use of pesticides and food additives.

As we have seen, a normal healthy individual consuming a reasonably varied diet composed naturally of thousands of toxic chemicals is not poisoned by it. The safety of our foods under these conditions is based on the assumption that, in the context of the total diet, each toxic chemical is present at a "no-adverse-effect" level. However, there are three general types of abnormal circumstances under which injury has been caused by the chemicals that occur naturally in foods:

- The seafood (Schantz, 1973) and microbial toxins (Wilson and Hayes, 1973), and the cardioactive glycosides in honey (Patwardhan and White, 1973) are categorized as *abnormal* (though natural) contaminants that adversely affect the *normal* consumer eating *normal* amounts of the foods in question.

- The goitrogens, lathyrogens, cyanogenetic glycosides, and avidin (Bell, 1973; Conn, 1973; Somogyi, 1973; VanEtten and Wolff, 1973) are *normal* constituents of foods and have caused disease in *normal* people consuming *abnormal* amounts of the foods in which they are present.

- Numerous *normal* components of foods consumed in *normal* amounts are harmful in *abnormal* individuals who have increased susceptibilities associated with diseased states, malnutrition, allergic sensitivities, inborn errors of metabolism, or nonspecific intolerances (Coon, 1973).

Examples of problems arising from this third type of abnormal circumstance are abundant. Even ordinary dietary levels of sodium, for example, are considered undesirable in hypertensive patients (Meneely, 1973). Wilson's disease, a hereditary defect in copper metabolism, can develop from normal intake of dietary copper (Underwood, 1973). Lactose intolerance (Patwardhan and White, 1973), gluten sensitivity (Jaffé, 1973), favism (Patwardhan and White, 1973), phytanic-acid-storage disease (Mattson, 1973), and phenylketonuria (Harper, 1973) are related to genetic defects in metabolism in which the afflicted individuals cannot tolerate normal dietary levels of specific natural chemical entities in foods which most people can eat with no adverse effects. (Scriver [1971] provided much insight into the human health significance of hereditary metabolic diseases and their control.)

From this, it can be appreciated that either abnormal contaminants, abnormal quantities of intake, or abnormal health or physiological makeup of the individual consumer can reveal the toxic potential of numerous natural chemical components of foods.

Evaluation of Natural Food Hazards

In spite of an occasional assumption to the contrary, plants and animals that have historically served as sources of food for man were not designed by nature for that purpose. Man discovered for himself, by trial and error, what he could eat with safety. Over the years, he discarded things that tasted bad or made him sick. Undoubtedly, most of his decisions to discard were based on what we would now call acute experiments; the injurious effects occurred so soon after eating that there was little question about what caused them.

However, long-delayed, harmful effects of repeatedly eating certain natural products remained a mystery until relatively modern times. The relationships between goiter, lathyrism, favism, and ergotism and their specific dietary causes were slow in coming to light. New suspicions of slowly developing or long-delayed effects have arisen in the light of new knowledge. It is now suspected, for example, that dietary sodium and excessive dietary cadmium play roles in the pathogenesis of hypertension.

Numerous substances that occur naturally in plant food sources are known or suspected to be carcinogenic in animals (Miller, 1973). Among these are safrole and related compounds, estrogens, antithyroid compounds, lead, and some of the fungal toxins as natural contaminants. More recently, the discoveries of the potent carcinogens, nitrosamines and polycyclic aromatic hydrocarbons as natural constituents of some plants have intensified the concern that the incidence of cancer in the human population may be partly attributable to the natural sources of our foods. Added to such uncertainties is the further suspicion that undiscovered carcinogens must still be lurking among the thousands of known and unknown chemical compounds that occur naturally in our foods.

Because of the chemical complexity of our total food supply, it is too much to expect that cause-effect carcinogenic relationships can be discovered. Identification of a carcinogen in a food may be simple, but ascertaining its carcinogenicity under the conditions of *consumption* of the food in which it is found will be impossible in most cases. Under these conditions, efforts to remove from the food supply all carcinogens that are now present or may be discovered in the future would disrupt that food supply far out of proportion to any putative benefits that might be derived.

In any case, it is clear that the real challenge that we face is the question of the long-term chronic toxicity, or lifetime effects, of the known and still unknown natural chemical components of our foods. Of greatest importance is the determination of such effects that might result from ordinary patterns of consumption, since they would be expected to affect the largest number of people. Though the problem of carcinogenesis has been emphasized above, similar attention should be focused upon reproductive functions, mutagenesis, cardiovascular-renal diseases, mental disorders, and other chronic ills of mankind of which the causes are unknown.

Evaluation of Hazards from Intentional Additives

Earlier, we alluded to relationships between the natural chemical components of foods and the chemicals entering foods as a result of man's efforts to improve the quantity and quality of his food supply.

In view of the current public concern about food contaminants (additives, pesticides, pollutants) and the relative absence of concern about the natural chemical composition that constitutes more than 99.5% of our dietary intake, these two categories of substances should be examined further in relation to each other.

More than 2,000 different substances are used as food additives. Though relatively few of these have been subjected to full-scale toxicological testing, many of them have been scientifically evaluated for safety under the conditions of prolonged use. Others have been accepted on the basis of the long history of their use. This concept has been applied in the case of saccharin, which has been used extensively in many countries for 80 years with no apparent harm to the consumer. As a synthetic chemical, however, saccharin recently became subject to a more intensive scientific scrutiny of its safety than is usually considered necessary for food additives that are natural products.

Safrole was used as an added flavoring agent in root beer until it was found to be a weak carcinogen in rats; however, this substance is naturally present in several spices still in wide use. The finding that vinyl thiooxazolidone and related compounds are potent goitrogens and are readily formed from precursors naturally present in cabbage, broccoli, and turnips has not influenced the growing or consumption of these vegetables. Thus, it is apparently considered safe to eat safrole and vinyl thiooxazolidone when they occur naturally in foods, but either of these agents would be considered unacceptable as a food additive.

If a natural chemical component of a food were found to have a desirable property as a food additive while its biological activity was still unknown, it would now have to be subjected to the same safety evaluation procedures required of a new synthetic chemical compound. Thus, whereas a food or an additive that has had a long history of use with no evidence of harmful effects is usually considered safe until proved harmful, a new food or additive, or even an old additive used in a new way, is considered harmful until proved safe.

On superficial grounds, it might be asked why we should be so concerned about the relatively small amounts of chemicals added to our food supply as a result of production, processing, and pollution activities, when there are so many known and unknown toxic substances naturally present in our foods. Obviously, the presence of such an abundance of toxic chemicals is reason enough to exercise caution in allowing the entry of any more noxious agents into the food supply.

The "safety in numbers" concept described previously does not take care of all contingencies, especially when a toxic chemical entering the food is the same as one already naturally present. For example, the fortification of foods with iron or vitamin D has been the subject of much controversy. In the area of pollution, the contamination of fish by mercury in industrial waste might lessen or even obliterate the margin of safety of this toxic element as it is naturally present in the fish.

Interactions of Toxicants Affect Hazard

It is unlikely, of course, that any of man's synthetic food additives or food-crop pesticides would add in this direct way to natural components of foods. There is the possibility, however, that such agents may participate in additive, synergistic, or potentiative toxicologic interactions with natural chemical constituents of foods. For example, the organophosphate insecticides are potent inhibitors of cholinesterase and would theoretically be expected to act additively with the cholinesterase inhibitors known to be present in a variety of common foods (Whitaker and Feeney, 1973). No practical hazard based on this situation, however, is known to exist, nor have there yet been found any other definite dietary hazards based on toxicologic interactions between natural food components and man's food additives or pesticides.

Indeed, there is reason to believe that the *antagonistic* or protective type of interaction between natural and added food chemicals may be much more common than the *synergistic* type. For example, DDT and most of the other organochlorine insecticides promote the production of enzymes in the body that detoxify many other chemicals, including some of the organophosphate insecticides (Bass et al., 1972). The pesticides pyrethrum (Springfield et al., 1971) and piperonyl butoxide (Wagstaff and Short, 1971) have similar enzyme-inducing properties. Nitrites that may arise from nitrates in foods produce a methemoglobinemia that detoxifies the cyanide generated from the cyanogenetic glycosides such as those found in lima beans. Gossypol in cottonseed has recently been found to promote the detoxification of some carbamate insecticides (Abou-Donia and Dieckert, 1971). Lindane has been reported to antagonize the depressant effect of lead on hemoglobin levels in mice (Cress and Larson, 1970). Numerous examples of antagonistic relationships between trace elements (Underwood, 1973), amino acids (Harper, 1973), and vitamins A and D (Hayes and Hegsted, 1973) have been described.

However, as in the case of the additive or synergistic toxicologic interactions among food chemicals, there is yet no instance in which an experimentally demonstrated antagonistic interaction between a natural food chemical and one added by man has been shown to be of practical significance for man in diminishing any potential hazard in his diet.

Since one chemical may influence the extent to which another chemical affects the toxicologic interaction between two other chemicals, it becomes readily apparent that the problem of potential toxicologic interactions is one of innumerable combinations of a multitude of compounds, many perceived only incompletely.

Margins of Safety Apply to *All* Components

When a chemical substance is proposed for use as a food additive or

food-crop pesticide, the amount permitted to remain in food products is set by government regulation at such a level that its estimated percentage in the total diet of the human consumer is equal to a small, specific fraction of the highest percentage in the diet that can be fed to test animals through their lifetime without any deleterious effect. A much practiced rule of thumb sets the acceptable fraction at one-hundredth of the no-adverse-effect level in the animal test diet, thus providing a 100-fold margin of safety.

The actual margin of safety applied for a given substance, however, may be more or less than 100, depending on the nature of the deleterious effect seen in animals fed high levels of the compound, on the scope of the toxicological information known about it, and on a judgment of the reliability of the data available. The bases on which these margins of safety are set have been discussed by FAO/WHO (1967).

Much more attention is given to the margins of safety of food additives and pesticides than to those of chemical substances that occur naturally in foods. The latter have been commonly accepted, not on the basis of scientific tests but because man has learned by experience that he is not hurt when he consumes these materials in the context of the ordinary diet. Very few natural chemical constituents of foods have been tested in animals in such a way that numerical margins of safety for lifetime consumption by man can be stated. In fact, it is doubtful that any broadscale effort to do this would be productive. If a substance usually consumed in its natural human food "habitat" were to be inserted into an animal food for a toxicity study, the opportunities for toxicologic interactions with other substances might be altered and its potential toxicity accordingly altered. The toxicologic balances seen in the cases of cadmium and zinc, copper and molybdenum (Underwood, 1973), selenium and mercury (Ganther et al., 1972), vitamins A and D (Hayes and Hegsted, 1973), and oxalic acid and calcium (Fassett, 1973) serve to illustrate this principle.

Past experience in man has contributed much more to our knowledge of margins of safety of natural foods than has animal experimentation, though there have been few efforts to express such margins for specific chemical components in quantitative terms. A wide variety of common foods contain known goitrogenic substances or antithyroid activity due to unknown components. Here the margin of safety, assuming a normal well-balanced diet on a chronic basis, is undoubtedly less than 10. A similar estimate for estrogenic activity could probably be made.

It is likely that the daily dietary intake of numerous elements such as iron, zinc, copper, and fluorine could not be increased by even 5 or 10 times without adverse effects on many people. From data presented by Meneely (1973) and Patwardhan and White (1973) from observations in both the rat and man, the margin of safety for sodium chloride in the diet, considering the differences between required and hypertensigenic amounts, may be less than 5, though the response in this respect appears to be conditioned to a great extent by hereditary factors and disease. It is well known that the margin of safety of sodium chloride in the hypertensive segment of the population is less than 1.

Another example is sugar, which has a very narrow margin of safety in the diabetic. The margins for vitamins A and D, in relation to what are considered daily requirements, appear to be about 25-40 in adults and possibly lower than 10 for infants or very young children (Hayes and Hegsted, 1973).

The real difficulty in making reliable estimates of margins of safety for prolonged consumption of toxic substances from observations on man is based on the lack of true chronicity in scientifically controlled tests. It is obviously not possible to subject human subjects to well-controlled testing procedures for a major part of their lifetime, as is done with experimental animals. Epidemiologic approaches must be considered more feasible.

In any case, it is likely that on a truly long-term basis, the above estimates of the margins of safety for various natural food components would be lower than those cited. Thus it is seen that the margins of safety for natural components of our daily diet are in many cases no greater, and in some cases considerably lower, than those legally allowed for food additives and pesticides.

Bodies Metabolize Molecules, Not Foods

Contrary to what many laymen think, synthesized substances do not contain insidious toxic properties compared with their natural counterparts. Many of the vitamins, for example, and many important drugs originally derived from plants are now chemically synthesized to provide an adequate supply for health needs, and the effects—beneficial or toxic—are identical in the natural or synthetic forms.

A greater suspicion is commonly focused upon synthetic chemicals that are not known to occur in nature. This suspicion appears directed at the possible deleterious effect of such chemicals on the gene pool of man. The assumption is that a single mutation induced by such a chemical could ultimately alter the evolutionary process. In 1963, Muller said, "Today we human beings are exposed to a great number of substances not encountered by our ancestors, to which we therefore have not been specifically adapted by natural selection" (Anonymous, 1969). Though this statement cannot be refuted on the basis of present theoretical knowledge, one might equally well submit opposing speculation.

Man is not yet fully adapted to the *natural* chemical components of his foods. More than a hundred hereditary diseases are now known, but there is no reason to believe that there are more such diseases now than there were when man's environment was wholly natural.

It seems logical to suppose that if the natural chemical components of our common foods were screened, one by one, for their mutagenic effects, as high a proportion of them would demonstrate this property as would be found among the synthetics that man has added to his food.

Man has added very few totally new synthetic chemicals to his food supply in proportion to the number of different chemical substances that are there naturally. Many, if not most, of even these synthetics may be chemically similar or related to substances in our natural foodstuffs, many of which are still unknown. Considering the

great number and variety of chemical substances in natural foods, it is questionable whether man has synthesized anything to add to his diet that is entirely new or baffling to the defense mechanisms of the body, in the amounts used. There is no reason to believe, for example, that the detoxication mechanisms of the body are less efficient in handling unnatural synthetic chemicals than in handling the natural chemical components of foods.

Risks Must Be Balanced Against Benefits

The considerations used in balancing the benefits of using food additives and pesticides against the risks associated with their consumption differ in some respects from those involved in evaluating the benefits and risks associated with the natural chemical constituents of our foods.

The essential justification for the use of an additive in food processing—or of a pesticide that leaves a toxic residue in foods—must be some direct or indirect benefit to the food consumer. Furthermore, it is a basic principle that the risks assumed to arise from the use of such substances should diminish as the relative importance of the benefit it is designed to achieve diminishes. An increased nutritional value, an increased food supply, keeping quality, or a substantially decreased cost to the consumer are benefits that might justify a small degree of theoretical risk. An improvement in such superficial esthetic qualities of food as flavor, color, or consistency, therefore, will not justify the use of a substance having a known or likely risk.

On the other hand, there are innumerable substances in natural foodstuffs that may provide attractive flavors and colors or determine form and consistency, and an even larger number of naturally occurring chemical components that have no such functions and provide no known benefits whatever. Many of these substances are known to have toxic properties, but it is apparent that the risks are widely accepted. The goitrogens, estrogens, and carcinogens naturally present in widely used articles of our diet may be cited as examples.

Benefit-Risk Concept Also Applies to Nutrients

It is of interest to reflect upon the benefit-risk equation as applied to the essential nutrients in foods. Many of these nutrients have toxic properties, but, since they are indispensable to life, their use is accepted—some risk might be thought to be acceptable. We have seen, however, that among the vitamins and essential elements, no risk has been recognized as being attributable to the natural presence of these substances in foods. For example, even though vitamin A has been shown to be teratogenic in several species of animals (Robens, 1970)—the margin of safety for the hamster, for example, is less than 60—no risk has ever been claimed to be associated with the natural amounts of this vitamin in foods, except in the liver of the polar bear and certain large fishes (Hayes and Hegsted, 1973).

When certain essential nutrients are used as food additives, the balancing of benefits and risks becomes important. The unquestioned benefits to many people of the fortification of foods with vitamin D or iron may be accompanied by an acceptable degree of risk to a few people. On the other hand, the use of sodium chloride as a food additive is not considered to be a benefit to health, even though sodium is an essential element naturally present in food. The potential risk of the intake of amounts of sodium only a few times greater than those naturally present in our foods has been extensively documented (Meneely, 1973).

Benefit-risk considerations are also encountered under various circumstances among nutrients supplying calories, the proteins and amino acids, fats and fatty acids, and certain disaccharides. Although many of the risks of these materials are based on inherited errors of metabolism in relatively few people, they are beneficial to the bulk of the population. For this type of benefit-risk problem, however, little can be done beyond the medical management and dietary control of the individual at risk.

Much broader implications for major segments of the population arise when one considers the benefits and risks of such things as sucrose and polyunsaturated fats. This has become important for these two substances because of the relatively recent increases in the amounts consumed, although there is no universal agreement as to the benefits or risks of either substance.

Safety Still Lies in Numbers —and Variety

The value of a varied diet has long been accepted as essential to consuming an adequate amount of essential nutrients for good nutrition. We have now further emphasized the value of a varied diet to ensure that the intake of specific chemical substances in foods will be insufficient to cause injury. This applies to natural chemical components of foods, to additives, and to contaminants, and it serves as the basis of the concept of "safety in numbers" referred to earlier.

Thus, public educational programs in nutrition should stress the use of a diverse and balanced diet as the basic approach to avoiding toxicologic hazard as well as to acquiring the nutritional essentials. Such teaching might provide some nutritional enlightenment for those who have been misguided into the hazards of the so-called Zen macrobiotic diet, the very antithesis of sound nutritional and toxicologic principles. Through a sequence of dietary regimens, it progresses to a diet made up 100% of cereals. It is not surprising that such deficiency diseases as scurvy, anemia, hypocalcemia, and others (even death) have been reported in the followers of this dangerous dietary philosophy (AMA Council on Foods and Nutrition, 1971; Stare, 1971).

Yet an extensively varied diet is not the definitive answer under all conditions to the question of the maximum safety of the foods man eats. First, numerous instances have been cited in which the margins of safety of certain natural components of foods are not wide enough to allow for even normally varied patterns of consumption; these instances include cases of greatly increased sensitivities associated with inborn errors of metabolism, allergic senstivities, indi-

vidual nonspecific intolerances, or a variety of disease states. Under some such conditions, it may be necessary to restrict the diversity of the diet, thus offsetting some of the advantages of a widely varied diet as consumed by individuals in good health and of normal constitution.

More Knowledge Needed on Natural Components

Second, there are extensive gaps in our knowledge of the significance to health of many of the substances known to be present in natural food products, as well as even the identity of many natural chemical components of foods and their potential toxicologic significance. The principal challenge we face in this regard is a knowledge of the long-term chronic effects on health of these naturally occurring compounds.

For example, are goitrogens, estrogens, safrole, and other known tumorigens of experimental animals responsible for any forms of malignant disease in man by virtue of their presence in many common food items? Do the sodium and cadmium contents of foods play a role in hypertension in man? What substances might be responsible for the development of various endocrine or mental disorders? These will be difficult problems to solve, complicated as they are by many interacting variables.

A clear obligation of the food and agricultural industries in their development and production of new or modified food products is to take into account the known chemical composition of the products they work with, especially in regard to those components that have toxic properties, whether or not such substances have yet been known to be harmful to man through his consumption of the foods containing them. The importance of this is illustrated by the recent finding that a new variety of potato, possessing improved chipping and browning properties, unfortunately contained an increased concentration of solanine alkaloids that prevented its further use (Whitaker and Feeney, 1973).

An awareness of the toxic properties of essential nutrients and of the amounts present in foods being processed should also be maintained by the food industry so that it may avoid the supplementation of its product with hazardous amounts of these agents.

Breeding and Processing Methods Need More Study

It is also highly desirable to investigate methods of selective breeding, to reduce the levels of toxic substances in plant food sources where potential hazards exist. By such methods, the toxic erucic acid has been practically eliminated from a rapeseed oil derived from the rape produced in Canada (Mattson, 1973), and cottonseed free of gossypol has been developed (Singleton and Kratzer, 1973). Selective breeding of the lima bean to reduce cyanogenetic glycoside content has also been encouraged, and the variety of the lima bean grown in the United States or permitted to be imported has a relatively low cyanide-generating capacity (Conn, 1973). Attention should clearly be given in such work to the associated changes that might occur in the food product, producing undesirable increases in the contents of other toxic components or decreases in the nutritional quality.

It is unlikely that it would be either feasible or beneficial, as a general approach in the processing of agricultural food products, to attempt to extract or remove many of the known toxic components. Such procedures would undoubtedly reduce the consumer acceptability, induce chemical changes that might introduce other toxic substances, or reduce the nutritional quality, thereby necessitating extensive animal studies to establish the safety of the products for prolonged consumption.

Some simple processes have long been used to render natural products safe or acceptable. Cooking destroys the cyanogenetic glycosides and goitrogens of various plant products, thiaminase in fish, avidin in the egg, and the hemagglutinins and enzyme inhibitors in beans. Native populations in the tropics have removed the toxic glucoside, cycasin, from the cycad nut by a water extraction procedure (Miller, 1973). In areas where cassava is an important food plant, a water-soaking and fermenting process has been effective in removing most of the cyanogenetic glycoside, linamarin (Conn, 1973).

We Must Learn More About *All* Components

Viewing all chemicals that are present in our food supply in perspective—the natural components, agricultural chemicals, food additives, and natural and man-made contaminants—it is clear that the greatest area of the unknown involves the normal and natural components of our foods. To achieve a more appropriate balance in the effort applied to the evaluation of the safety of foods in general, it is reasonable that at least as much attention should be given to these substances as is given to the additives and pesticide residues. This is not to suggest that less attention be given to the latter. In fact, due consideration should be given to the potential problems that might arise from the simultaneous presence of these two groups of chemicals and the resulting chemical and toxicologic interactions between them, as well as with those miscellaneous contaminants in food.

As far as man's food intake is concerned, the ultimate goal should be to gain an understanding not only of what constitutes the optimum in nutritional content, but also of what involves the minimum of long-range, lifetime toxicologic hazard in the diet.

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