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Apologia

Changes in format, typography, handling (and a new printer with a striking work force—for a while) have caused delays in publication of the *Journal*. We ask your forbearance. At this time it is expected that issues will be mailed on schedule beginning with No. 2—1970.

ABSTRACTS:
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CAPILLARY DISTRIBUTION AND FIBER CHARACTERISTICS IN SKELETAL MUSCLE OF STRESS-SUSCEPTIBLE ANIMALS. C. C. COOPER, R. G. CASSENS & E. J. BRISKEY. *J. Food Sci.* **34,** 299–302 (1969)—More capillaries were associated with red fibers than with white fibers and there was a greater capillary fiber ratio in red than white muscle. There was no difference in capillary to fiber ratio between normal (from stress-resistant pigs) and PSE (from stress-susceptible pigs) muscle. Muscle from stress-susceptible animals had more intermediate fibers than did muscle from stress-resistant animals. Certain intermediate fibers from stress-susceptible animals had a high amylophosphorylase and ATPase activity. It was postulated that such fibers are important in the drastic post-mortem muscle behavior in stress-susceptible animals and may represent the characteristic which causes muscle to become PSE.

TURBIDITY, VISCOSITY AND ATPase ACTIVITY OF FIBRILLAR PROTEIN EXTRACTS OF RABBIT MUSCLE. P. D. WEINER, A. M. PEARSON & B. S. SCHWEIGERT. *J. Food Sci.* **34,** 303–305 (1969)—ATPase activity, turbidity and viscosity were determined for fibrillar protein extracts from prerigor and rigor rabbit muscle. Results suggest that actomyosin from rigor muscle is more easily dissociated than actomyosin from prerigor muscle. Addition of $MgCl_2$ decreased the rate of ATP hydrolysis and extended the clear phase. Pyrophosphate plus magnesium was just as effective as ATP for clearing actomyosin. On hydrolysis of ATP, viscosity increased to values greater than before the addition. However, pyrophosphate-cleared samples retained their low viscosity indefinitely.

CONNECTIVE TISSUES FROM NORMAL AND PALE, SOFT AND EXUDATIVE (PSE) PORCINE MUSCLES. 2. Physical Characterization. P. E. McCLAIN & A. M. PEARSON. *J. Food Sci.* **34,** 306–308 (1969)—The epimysial connective tissues from normal and PSE longissimus dorsi muscles were subjected to physical characterization. No differences in conventionally determined shrinkage temperatures were observed. However, differential thermal analysis (DTA) studies revealed that epimysium from PSE tissues had lower onset and recovery temperatures and contained a higher percentage of components melting at low temperatures than epimysium from normal tissues. The epimysium from PSE pigs also had a higher initial moisture and lower dry matter content. Epimysial connective tissues underwent osmotic swelling in neutral solutions, with those from PSE muscles imbibing significantly more water than those from normal muscles.

STUDIES ON NATURAL ACTOMYOSIN: SURVEY OF EXPERIMENTAL CONDITIONS. H. K. HERRING, R. G. CASSENS, T. FUKAZAWA & E. J. BRISKEY. *J. Food Sci.* **34,** 308–311 (1969)—Procedures for the preparation of natural actomyosin are given along with a comparison of some properties of rabbit and bovine actomyosin. Rabbit actomyosin had about two times the ATPase activity of bovine actomyosin. Actomyosin from both species behaved similarly with respect to ionic conditions.

EFFECT OF DIETARY FATS ON SOME CHEMICAL AND FUNCTIONAL PROPERTIES OF EGGS. R. D. PANKEY & W. J. STADELMAN. *J. Food Sci.* **34,** 312–317 (1969)—Fatty acid composition of total yolk lipids (triglyceride, cepholin and lecithin fractions of lipovitellin and lipovitellenin) was influenced by 10% vegetable oil diet supplement (corn, soybean, olive, safflower or hydrogenated coconut oil). The fatty acid composition of the total yolk lipids was influenced by all dietary fats. The major change was in the linoleic acid at the expense of oleic acid with corn, soybean and safflower oil. Olive oil increased the oleic acid and hydrogenated coconut oil increased lauric, myristic and myristoleic acids. The fatty acid composition of the fractions of the lipoproteins was influenced by the dietary fats and varied between fractions.

POTENTIOMETRIC DETERMINATION OF FLUORIDE IN BEVERAGES BY MEANS OF THE ION SELECTIVE SOLID STATE ELECTRODE. W. P. FERREN & N. A. SHANE. *J. Food Sci.* **34,** 317–319 (1969)—Free available fluoride ion content of representative carbonated and noncarbonated beverages was determined in the presence of a buffered "swamping solution." Results indicated (1) commercial carbonated beverages analyzed had fewer than 0.019 ppm fluoride, (2) noncarbonated beverages prepared at home from packaged powders retain the free available fluoride present in the fluoridated water supplies, (3) milk samples and orange juice samples analyzed had fewer than 0.019 ppm for free available fluoride but when spiked with sodium fluoride, the fluoride present is in the free available form.

LETHALITY OF RADIO-FREQUENCY ENERGY UPON MICROORGANISMS IN LIQUID, BUFFERED, AND ALCOHOLIC FOOD SYSTEMS. D. E. CARROLL & A. LOPEZ. *J. Food Sci.* **34,** 320–324 (1969)—This work was done to determine if 60 mc/sec R-F energy has a selective killing effect on microorganisms other than that attributable to heat. The organisms studied were *Saccharomyces cerevisiae, Escherichia coil* and *Bacillus subtilis.* No killing effect of the R-F energy per se on the organisms was observed at any of the various buffer pH values, nor was there an observable synergistic killing effect of R-F energy and heat on the microorganisms. However, a synergistic killing effect of ethanol and heat at 48.8°C was demonstrated on *S. cerevisiae.* Irradiating *S. cerevisiae* and *E. coli* in several liquid foods also failed to show a selective killing effect of R-F energy.

REACTION AT LIMITED WATER CONCENTRATION. 1. SUCROSE HYDROLYSIS. T. SCHOEBEL, S. R. TANNENBAUM & T. P. LABUZA. *J. Food Sci.* **34,** 324–329 (1969)—Saturated sucrose solutions containing various acids and inert solid materials gave identical rate constants and energy of activation as predicted from dilute solutions. Reaction rates in freeze-dried systems humidified to low moisture contents indicated that any equation describing the rate of hydrolysis must include a term for the velocity of dissolution of solid sucrose into the surface water. The rate of hydrolysis was a pseudo first-order reaction obeying the same kinetics as in dilute solution; the rate of dissolution became rate-limiting when the initial supply of dissolved sucrose was exhausted.

FREE AMINO ACIDS IN BOVINE MUSCLES AND THEIR RELATIONSHIP TO TENDERNESS. R. A. FIELD & YET-OY CHANG. *J. Food Sci.* **34,** 329–331 (1969)—Free amino acid (FAA) analyses were conducted on 87 l. dorsi and 58 b. femoris samples from cattle varying in sex, breeding and age to study the relationships between these muscle constituents and Warner-Bratzler Shear values. The b. fermoris contained greater amounts of FAA than the l. dorsi and was significantly tougher. Muscles from a line of cattle that tended to be more tender possessed greater amounts of FAA. With few exceptions the l. dorsi of steers contained greater amounts of FAA than the same muscle from bulls. Differences in bull muscles due to age were not significant for any FAA. Steer muscle with low shear values tended to possess greater amounts of FAA than steers with high shear values. Although not significantly correlated, individual and total FAA increased slightly with increasing tenderness within beef muscles.

SOME PROPERTIES OF MITOCHONDRIA FROM IRRADIATED TOMATO FRUIT. S. R. PADWAL-DESAI, E. M. AHMED & R. A. DENNISON. *J. Food Sci.* **34,** 332–335 (1969)—Homestead tomato fruit were irradiated with 300 Krad of ^{60}Co gamma rays at three stages of maturity—mature-green, 2 days and 6 days from color break. Though oxygen consumption by mitochondrial preparations was greatly suppressed immediately after irradiation, it increased to a maximum during the following 2 days and then declined similarly to nonirradiated fruit. Oxygen consumption was reduced considerably during ripening of the nonirradiated and irradiated 6 day breakers. Mitochondria from irradiated fruit exhibited lower P/O ratios than from nonirradiated fruit. A considerable reduction in mitochondrial protein, was found during ripening of mature-green fruit. This reduction was less apparent with the 2 and 6 day breakers.

THE VOLATILE COMPONENTS OF IRRADIATED BEEF AND PORK FATS. J. R. CHAMPAGNE & W. W. NAWAR. *J. Food Sci.* **34,** 335–339 (1969)—Of the components produced by irradiation, 41 were identified by gas chromatography and mass spectrometry. These include a series of *n*-alkanes, 1-alkenes, internally unsaturated alkenes and alkadienes. The latter two groups of compounds have not been reported previously in meats or meat fats. In addition, hexadecanal, octadecanal and octadecenal were found to be produced in relatively large quantities by irradiation. Flavor threshold values of the unsaturated classes of hydrocarbons vary widely among themselves but are much lower than those of the *n*-alkanes.

IDENTIFICATION AND CHARACTERIZATION OF THE PECTIC ENZYMES OF THE MCFARLIN CRANBERRY. O. A. ARAKJI & H. Y. YANG. *J. Food Sci.* **34,** 340–342 (1969)—Endo-polygalacturonase and pectin esterase were found in frozen cranberries. Phenol binding agents were necessary to obtain high enzyme activity. Optimum pH's for cranberry polygalacturonase and pectin esterase were 5.0 and 7.5, respectively. Heating at 100°C destroyed polygalacturonase in 35 min and esterase in 5. Optimum pectin esterase activity occurred when treated with 0.15 *M* NaCl.

THE RELEASE OF DIPICOLINIC ACID FROM SPORES OF Bacillus stearothermophilus NCA 1518. Y. ROTMAN & M. L. FIELDS. *J. Food Sci.* **34,** 343–344 (1969)—Both pH of the spore suspensions and autoclaving time affect the release of dipicolinic acid (DPA) from spores. At pH 14 maximum release of DPA was obtained for spore suspensions of both variants autoclaved at 250°F for 15 min. With the smooth variant, maximum release of DPA was also achieved at pH 7.0. Autoclaving at 250°F for 15 and 70 min was required for complete release of DPA from spores of the smooth and the rough variants. Loss of viability of spores of both variants succeeded complete release of DPA.

CHEMICAL COMPOSITION AND HEAT RESISTANCE OF Bacillus stearothermophilus SPORES. Y. ROTMAN & M. L. FIELDS. *J. Food Sci.* **34,** 345–346 (1969)—Heat resistance measurements demonstrated that the rough variant was more heat tolerant than the smooth variant. Spores of the two variants were analyzed for DPA, calcium, manganese, magnesium and zinc. No direct relationship was found between DPA, mineral concentrations and heat resistance.

A STUDY ON THE SPORULATION OF ROUGH AND SMOOTH VARIANTS OF Bacillus stearothermophilus. Y. ROTMAN & M. L. FIELDS. *J. Food Sci.* **34,** 346–349 (1969)—Sporulation of the two variants, rough and smooth, of *B. stearothermophilus* NCA 1518 was studied in three complex media: nutrient agar, nutrient broth and trypticase soy agar. The rough variant sporulated best on nutrient agar enriched with one ppm manganese with or without yeast extract, but did not sporulate in liquid media. Aerated broth fortified with yeast extract and 1 ppm manganese was the best sporulating medium for the smooth variant. The effects of calcium, cobalt and dextrose on the sporulation of the two variants are discussed.

COLORIMETRY OF FOODS. 1. Correlation of Raw, Transformed and Reduced Data with Visual Rankings for Spinach Puree. F. M. CLYDESDALE & F. J. FRANCIS. *J. Food Sci.* **34,** 349–352 (1969)—Color data were obtained for three series of processed, stored spinach purées using a General Electric Recording Spectrophotometer, a Hunterlab Model D25 Color Difference Meter, and a Colormaster Differential Colorimeter, Model V. Tristimulus values from the three instruments were mathematically transformed to the tristimulus values of other two color systems in each case. Tristimulus values from the instruments were also reduced to common color functions. Correlations of these data versus visual ranking of the samples were also calculated. Results indicated good correlation of raw instrument data with visual rank but transformation or reduction of the data often lowered the correlations.

DETERMINING GLUCOSE AND GLYCOGEN FROM A SINGLE SAMPLE OF MEAT. P. M. HEFFERAN & K. C. GOODNIGHT. *J. Food Sci.* **34,** 353–354 (1969)—A method was developed determining glucose and glycogen as separate entities from a single sample of meat. This method is based on the quantitative separation of glycogen from glucose by precipitation of the former species with an inert organic solvent. During this separation care must be exercised not to allow the temperature of the meat extract to rise above 5°C. After separation the two carbohydrate species may be analyzed by existing methods. The accuracy of this method was tested by comparing its results with those of established methods for the determination of glucose and glycogen.

NUTRITIVE VALUE OF 1,2-DICHLOROETHANE-EXTRACTED FISH PROTEIN CONCENTRATE. B. H. ERSHOFF & P. G. RUCKER. *J. Food Sci.* **34,** 355–359 (1969)—The nutritive value of fish protein concentrate (FPC) preparations was dependent on the temperature and length of extraction time. Rats fed diets containing FPC extracted at 65° ± 2°C or 40° ± 2°C for 24 hr or at a temperature of 83°C for 6 hr or 3 hr and vacuum dried without steaming were normal in appearance and exhibited increments in body weight and feed efficiency comparable to those of rats fed similar 20% casein protein diets. FPC extracted for 24 hr at 83°C without replacement of evaporated solvent during the last 6 hr of extraction was toxic.

QUANTITATIVE DETERMINATION OF FORMIC, ACETIC, PROPIONIC AND BUTYRIC ACIDS IN FROZEN WHOLE EGGS BY GAS-LIQUID CHROMATOGRAPHY. J. E. STEINHAUER & L. E. DAWSON. *J. Food Sci.* **34,** 359–364 (1969)—Whole eggs containing known amounts of formic, acetic, propionic and butyric acids (C_1–C_4) were evaluated using gas-liquid chromatography (GLC) and AOAC (1960) procedures. Acetic, propionic and butyric acids were recovered and chromatographed as the acids per se, and formic and acetic acids as their butyl ester derivatives. The recovery of acids from whole egg samples by GLC procedures was as accurate, and generally less variable, than by AOAC (1960) procedures. The percentage recovery depended on the concentrations of the acids present, and was as follows, low to high concentrations respectively: acetic acid, 114 to 100%; propionic acid, 101 to 100%; butyric acid, 103 to 99%; formic acid (as butyl ester), 104 to 96%; and acetic acid (as butyl ester), 102 to 98%.

THE ANTHOCYANINS OF STRAWBERRY, RHUBARB, RADISH AND ONION. T. FULEKI. *J. Food Sci.* **34,** 365–369 (1969)—The presence of a large number of previously unnoticed anthocyanins was demonstrated in strawberry, rhubarb, radish and onion. The number of anthocyanins found in this survey and those reported previously (in parenthesis) in each plant were as follows: strawberry: 6 (4), rhubarb: 3 (2), radish: 13 (5), onion: 8 (3). Some of the chromatograms indicated that the number of anthocyanins present was even greater than that. A quantitative rather than qualitative difference was found between the anthocyanin pattern of the examined varieties. The survey was carried out using paper chromatography on Whatman No. 3 MM paper with two new solvent systems of high resolving power. The solvents were: 1-butanol-benzene-formic acid-water (100:19:10:25) and 1-butanol-formic acid-water (100:25:60). The factors which improved the sensitivity of the chromatographic method employed were discussed in some detail.

ENZYME-CATALYZED REACTIONS AS INFLUENCED BY INERT GASES AT HIGH PRESSURES. J. R. BEHNKE, O. FENNEMA & W. D. POWRIE. *J. Food Sci.* **34,** 370–375 (1969)—The activities of various enzymes were studied after exposure to different high-pressure inert gases. Exposure to these gases failed to significantly inhibit tyrosinase activity in fluid systems, but significantly depressed the rates of tyrosinase-catalyzed reactions in nonfluid systems. This inhibition was oxygen dependent and reversible. Pressurization experiments with invertase, trypsin and chymotrypsin indicated that high-pressure inert gases did not significantly inhibit these nonoxygen dependent enzymes. There appears little hope that the enzymes in food systems can be effectively inhibited by brief exposure to inert gases at pressures of 5000 psig or less.

THE INFLUENCE OF TENSION ON PRE-RIGOR EXCISED BOVINE MUSCLE. W. A. GILLIS & R. L. HENRICKSON. *J. Food Sci.* **34,** 375–377 (1969)—The influence of four levels of tension in two bovine muscles on fiber diameter, sarcomere length, percent kinkiness, and shear value were studied. Fiber diameter was found to be smaller with 1,000-g pull treatment than when there was no tension, but no significant change was found with succeeding increases in tension. Sarcomere length was found to increase to the 2,500-g pull treatment and then level off. Both muscles exhibited less kinkiness to the 1,000-g pull treatment. However, after this level the semitendinosus muscle increased whereas the semimembranosus decreased.

ENZYMIC AND ACID HYDROLYSIS OF SUCROSE AS INFLUENCED BY FREEZING. D. B. LUND, O. FENNEMA & W. D. POWRIE. *J. Food Sci.* **34,** 378–382 (1969)—Sucrose hydrolysis catalyzed by invertase was studied over the temperature range 12 to −22°C in solutions of various concentrations. Freezing decreased invertase activity, but it was shown that ice crystals were not directly responsible. The decrease in invertase activity in frozen systems could be quantitatively accounted for by the concentration of solutes resulting from freezing, and by a temperature effect in excess of that predicted from the Arrhenius equation. Sucrose hydrolysis catalyzed by acid (HCl) was studied over the range 12 to −16.5°C. The reaction rate passed through a sub-zero maximum between −8.5 and −16.5°C.

HIGH RESOLUTION VAPOR ANALYSIS FOR FRUIT VARIETY AND FRUIT PRODUCT COMPARISONS. R. A. FLATH, R. R. FORREY & R. TERANISHI. *J. Food Sci.* **34,** 382–386 (1969)—By using a system incorporating large-bore open-tubular gas chromatographic columns, high resolution vapor analyses of five fresh apple varieties and four apple products were run. The improved peak separation attainable with such a system permits more detailed comparisons between different samples. This is a considerable aid in varietal studies and in following loss and change of volatiles during processing. The apple varieties examined appear to differ mostly in the relative proportions of individual components rather than in the presence or absence of certain constituents.

DEGRADATION OF DDT AND DDE BY CHEESE MICROORGANISMS. R. A. LEDFORD & J. H. CHEN. *J. Food Sci.* **34,** 386–388 (1969)—Growth of Liederkranz cheese isolates of Brevibacterium linens and species of geotrichum in media containing 0.5 ppm lindane, p-p DDE, and p-p DDT resulted in significant reductions in concentrations of DDE and DDT. Aerobic metabolism of B. linens appeared to dechlorinate DDT to DDD, while growth of geotrichum degraded both DDE and DDT. The level of lindane in all experiments was not changed significantly. The data suggest the possibility that DDE and DDT, if present in certain types of soft cheeses, might be degraded to unidentified compounds.

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Capillary Distribution and Fiber Characteristics in Skeletal Muscle of Stress-Susceptible Animals

SUMMARY——Muscle from stress-susceptible and stress-resistant pigs was studied for capillary distribution and fiber characteristics. More capillaries were associated with red fibers than white fibers and there was a greater capillary to fiber ratio in red than white muscle. There was no difference in capillary fiber ratio between normal (from stress-resistant pigs) and PSE (from stress-susceptible pigs) muscle. Muscle from stress-susceptible animals had more intermediate fibers than did muscle from stress-resistant animals. Certain intermediate fibers from stress-susceptible animals had a high amylophosphorylase and ATPase activity. It was postulated that such fibers are important in the drastic post-mortem muscle behavior in stress-susceptible animals and may represent the characteristic which causes muscle to become PSE.

INTRODUCTION

LACTIC ACID levels in the muscles of "stress-susceptible" (Judge et al., 1968) animals increase rapidly when muscles are exposed to anoxia in vivo (Forrest et al., 1968; Sair et al., 1969) and the animals will succumb if not given therapy. Lactic acid levels also increase rapidly due to muscle excision or animal exsanguination (Lister et al., 1969). In all instances, as large amounts of lactic acid accumulate, creatine phosphate (CP) levels diminish and adenosine triphosphate (ATP) breaks down rapidly. These rapid metabolic changes result in an extreme acidity at body temperature and the muscles develop a pale, soft, exudative (PSE) character.

Even when animals are anesthetized, intubated with endotracheal tubes and given positive pressure respiration during and after administration of *d*-tubo-curarine chloride, the concentration of CP and lactic acid in subsequent biopsy samples are especially variable (Lister et al., 1969). A considerable decrease in CP and increase in lactic acid may occur during the excision of a sample and prior to cessation of its metabolic activity by freezing in liquid nitrogen.

Lister et al. (1969) noticed a difference between muscles from stress-susceptible and stress-resistant animals during the course of routine histological observations. Sudan black B positive fibers were larger in diameter and represented a large proportion of the total fiber area in stress-susceptible animals. Since typical red fibers are known to have a low adenosine triphosphatase (ATPase) activity (Barany et al., 1965) and low phosphorylase activity (Bocek et al., 1966), it is difficult to explain why anoxia should produce a rapid increase in lactic acid level from muscles with a high red fiber content. Additionally, Quass et al. (1968) found that myosin isolated from muscles of stress-susceptible animals had higher Ca^{++}-activated ATPase activity than myosin from muscles of stress-resistant animals.

It appeared that muscles of such stress-susceptible animals varied in available blood supply or inherent metabolic differences of individual fibers. The present study represents a histochemical evaluation of the capillary supply to individual fibers, as well as specific characteristics of red, white and intermediate fibers from stress-susceptible animals which developed PSE muscle and stress-resistant animals with normal muscle. Particular emphasis was given to the histochemical evaluation of amylophosphorylase and ATPase activity.

MATERIALS & METHODS

THE stress-susceptible and stress-resistant pigs used in this study were from the same strains of Poland China and Chester White animals previously described by Judge et al. (1968) and Forrest et al. (1968). All animals were of approximately the same age and weight (90 kg) and were reared with identical environments and feeding regimes.

For the capillary study, the longissimus and trapezius muscles from eight pigs were used. Immediately after exsanguination, samples from both muscles were excised, frozen at liquid nitrogen temperature and then equilibrated at −20°C prior to sectioning. Serial sections, 10μ in thickness, were cut in a cryostat, mounted on slides and air dried prior to incubation in the appropriate histochemical media. A modified azo dye technique (Barka et al., 1963) was employed for staining capillaries with α-naphthyl phosphate and diazotate-4′-amino-2′, 5′-Diethoxybenzanilide.

The procedure was essentially that described by Romanul (1965). Amylophosphorylase, adenosine triphosphatase (ATPase) and reduced diphosphopyridine nucleotide-tetrazolium reductase (DPNH-TR) stains as described by Engel et al. (1967) were utilized in these studies. The capillary fiber ratio was determined as an average from 25 muscle bundles for each animal. Longissimus muscle from an additional six pigs was subjected to the amylophosphorylase, ATPase and DPNH-TR reactions in order to study the fiber type pattern in stress-susceptible and stress-resistant animals further.

RESULTS & DISCUSSION

Capillary distribution

Figure 1 illustrates the appearance of capillaries as distinguished with the alkaline phosphatase reaction. The reaction product reportedly occurs in the capillary endothelium (Romanul, 1965). Bare spots or patches of no reaction have also been considered a difficulty with this technique. However, we felt the technique worthy of investigation, particularly in view of the probable connection of the circulatory system to stress-susceptible animals and the PSE problem.

Experiments to perfuse the longissimus are also prone to some problems inherent in the methodology. Most of the capillaries were located axially within the fasiculi with only a few at the periphery of the fasiculi. There were also segments of capillary rings around some fibers located peripherally in the bundles. This could be interpreted to indicate that when the microcirculatory system enters the bundle it transverses the outer fibers until it reaches the axial portion of the bundle where it parallels the fiber. Upon leaving the fascicle the microcirculatory system once again transverses the outer fibers of the muscle bundle (Krogh, 1929).

When serial sections were incubated for the DPNH-TR reaction, the number of capillaries surrounding each fiber or fiber group appeared in proportion to its redness or its oxidative metabolic activity. In many cases, the only capillaries associated with white fibers appeared to be shared with adjacent red fibers. The stain intensity in the capillaries varied throughout each section. Both aspects are in agreement with the findings of Romanul

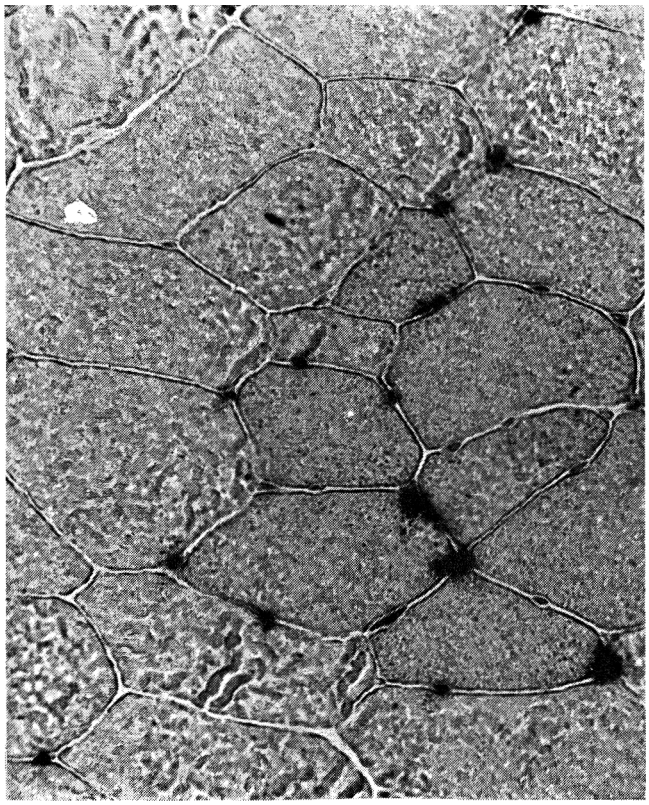

Fig. 1—Fresh frozen section of porcine longissimus muscle reacted for alkaline phosphatase. Capillaries are seen as dark reaction deposits. 10μ thick section, 265×.

(1965) that the number of detectable capillaries vary with fiber redness and that the degree of stain intensity appears less toward the venous ends of the capillaries.

Exercise, condition, acclimation and disease (Lindgren, 1935; Krogh, 1918–19; Ashmore et al., 1968; Carrow et al., 1967; Rakusan et al., 1967; Valdivia, 1958) all have a marked influence on vascularization. We therefore took precautions to standardize nutritional, holding and handling conditions.

No difference in capillary to fiber ratio was found between PSE and normal muscle, however the number of capillaries per unit area of red or intermediate fibers was less in the PSE muscle. The total capillary to fiber ratio was found to be approximately 1:3 in the longissimus and 1.2:1 in the trapezius. Although these figures seem low for the longissimus they compare favorably with the work of Nishiyama (1965) and Smith et al. (1956) with muscles of small laboratory animals. The greater supply of capillaries to red muscle which was expected was found.

It is well known that blood flow is normally under sensitive control in skeletal musculature and appears to be closely associated with metabolic activity within the fibers (Green et al., 1959). Romanul (1965) suggested that the existence in the skeletal muscles of some fibers with an energy metabolism similar to that of heart muscle may be a reflection of the fact that in skeletal muscles such fibers also contract more frequently or for longer periods than the rest of the fibers. Furthermore, according to Romanul (1965) the axial location in the bundle of fibers with high lipid metabolism would be expected if these fibers were to contract independently or if their contraction were to endure beyond that of other fibers. The white fibers which were dependent upon anaerobic glycolysis, were somewhat self-sufficient during contraction and appeared to depend on blood flow mainly for the removal of lactic acid. Conversely, the red fibers, which functioned through oxidative metabolism, were dependent upon circulating blood for substrate and oxygen necessary to produce energy for contraction.

Smith et al. (1956) and Romanul (1965) have demonstrated a greater supply of capillaries to red than white muscles. Additionally, Nishiyama (1965) has shown that the red, white and intermediate fibers individually present dissimilar patterns of vascular distribution. The fact that muscles of stress-susceptible animals which appear high in intermediate fiber content do not have grossly dissimilar capillary networks perhaps indicates another deviation from the norm in stress-susceptible pigs.

Fiber characteristics

Ranvier (1873, 1874) was one of the first workers to categorize muscle as either red or white. Denny-Brown (1929) and Ogata (1958) substantiated that muscle color was dependent upon the proportion of red fibers within the muscle. Red fibers have high oxidative enzyme activity, whereas white fibers have high glycolytic activity.

Within the past two decades, several workers have used histochemical techniques to classify fibers into red, white and intermediate groups. Padykula (1952) categorized fibers according to their metabolic functions as revealed by strong or weak reactions for succinate dehydrogenase activity. Dubowitz et al. (1960) used stains for succinate dehydrogenase, cytochrome oxidase and phosphorylase to demonstrate an inverse relationship between mitochondrial and glycolytic enzymes.

Recently Sair et al. (1969) studied fiber characteristics of PSE and normal pig muscle using Sudan black B, cytochrome oxidase and succinic dehydrogenase but they did not differentiate between red and intermediate fibers. These workers, (Sair et al. 1969) however, concluded that PSE muscle fibers were larger and that there was a larger area of red fibers (red and intermediate) per bundle in the PSE muscles than in the normal muscles. Kastenschmidt et al. (1968), in a study of "fast" and "slow" glycolyzing muscle, found that the metabolic intermediate patterns were consistent with the concept that phosphorylase was the primary control site for post-mortem glycolysis. A rapid breakdown of ATP and CP in the muscles of stress-susceptible animals was also substantiated by Sair et al. (1969).

In view of the reports of Kastenschmidt et al. (1968) that phosphorylase activity was higher in muscles of stress-susceptible animals and of Quass et al. (1968) that myosin ATPase activity was higher in PSE muscle it was particularly striking that Sair et al. (1969) found these muscles to have higher apparent red fiber areas. Theoretically, red fibers should have lower phosphorylase and ATPase than white fibers. Consequently, the present study was conducted to histochemically characterize the individual fibers within these muscles.

Figure 2 illustrates the histochemical staining pattern for longissimus muscle from a stress-resistant and a stress-susceptible animal. Figures 2a, b, c are from a stress-resistant animal and Figures 2d, e, f are from a stress-susceptible animal. The same group of fibers have been numbered in serial sections reacted for DPNH-TR, amylophosphorylase and ATPase in order to facilitate a discussion of the fiber characteristics from each classification of animals.

The first obvious difference between

muscle from stress-resistant (Figs. 2a, b, c) and stress-susceptible (Figs. 2d, e, f) animals is that stress-susceptible animals have larger fibers (Sair et al. 1969). This difference in fiber size from animals of the same body weight may be of straightforward significance to the PSE problem. We found no difference in capillary/fiber ratio for normal and PSE muscle; perhaps the larger fibers simply cannot be supplied or relieved of substances as rapidly as smaller fibers. Differential contraction may also be important.

The reaction to DPNH-TR should indicate oxidative potential of the fiber. The muscle from the stress-resistant animal (Fig. 2a) showed a more uniform deposition of diformazan than did that from the stress-susceptible animal (Fig. 2d). Red and intermediate fibers can be differentiated on the basis of intensity and uniformity of diformazan deposits. Heavy, uniform deposits are typical of true red fibers, whereas dense subsarcolemmal deposits with light centers are typical of intermediate fibers (see fibers 5 and 6, Fig. 2d).

The reaction for amylophosphorylase reveals an essential difference between the muscles of stress-resistant and stress-susceptible animals. Fibers 1, 2, 3, 4 and 5 (Fig. 2b) from the stress-resistant animal show a negative reaction for amylophosphorylase. However, fibers 1 and 2 and to a lesser extent 5 and 6 (Fig. 2e) from the stress-susceptible animal show a positive reaction for amylosphosphorylase even though they had given a definite reaction for DPNH-TR. A quite similar situation exists for the ATPase reaction (see fibers 1, 2, 5 and 6 in Fig. 2f.

This is completely contrary to the classical idea that fibers high in oxidative capacity (high DPNH-TR) are low in glycolytic capacity (low phosphorylase). Moreover, red fibers (high DPNH-TR) contract slowly and should have a low ATPase activity. It was found that many fibers which appear to be red by the DPNH-TR reaction are in reality intermediate as shown by the amylophosphorylase and ATPase reactions. Compared to stress-resistant animals, stress-susceptible animals have high numbers of these intermediate fibers, and we feel that the unusual post-mortem behavior of muscle from stress-susceptible animals is related to these fiber characteristics.

Animals with high stress-susceptibility, poor muscle color and poor gross morphology ratings were placed in one group, and those with high stress-resistance, desirable muscle color and desirable gross morphology were placed in another group in order to obtain more quantitative data. Several (6–8) adjacent microscopic fields were photographed and analyzed with a Zeiss Particle Size Analyzer for area distribution of fiber types (see Cassens et al. 1969a for details of method). The white fiber area expressed as a percent of total fiber area was rather constant between stress-susceptible and stress-resistant animals with a normal range of 70–75%. The remainder of the fiber area was made up of red and intermediate fibers, hereafter referred to as "dark" fibers.

In the muscle of stress-susceptible animals which became PSE in character, only 20–35% of the dark fibers were actually red, whereas in the case of stress-resistant animals with normal muscle, 60–75% of the dark fibers were considered red on the basis of their negative responses to phosphorylase and ATPase stains. It is clear that: 1) there were considerably fewer red fibers and more intermediate fibers in muscle from stress-susceptible animals which became PSE than in muscle from stress-resistant animals which remained normal in color and gross morphology, and 2) there were increased intensities of reaction for ATPase and phosphorylase in the muscle fibers from stress-susceptible animals which became PSE and this increased intensity was especially evident in the large intermediate fibers. It therefore appears that it is both the number and nature of the intermediate

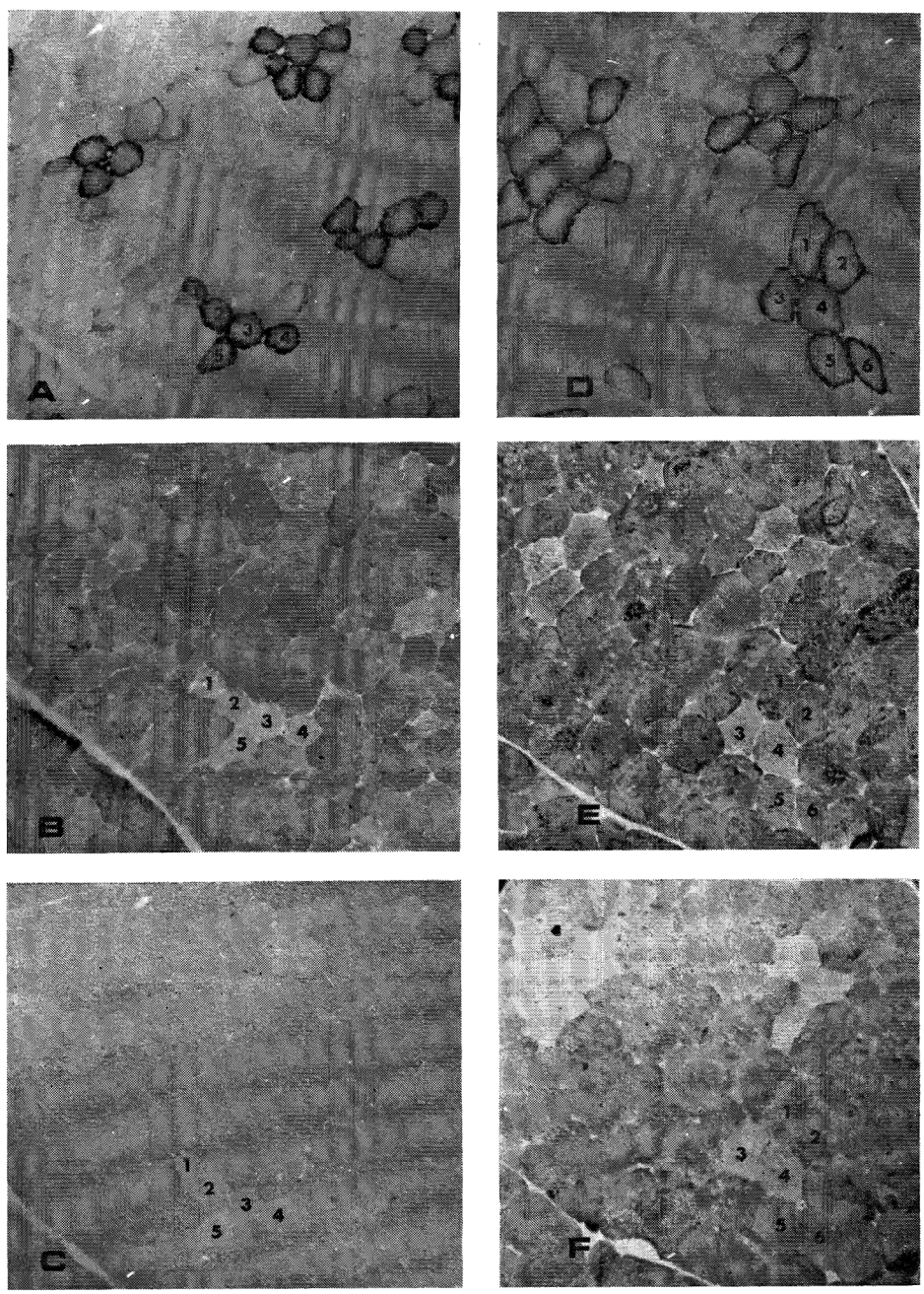

Fig. 2—Fresh frozen section of porcine longissimus muscle reacted for DPNH-TR (A&D), amylophosphorylase (B&E) and ATPase (C&F). A, B and C from stress-resistant animal and D, E and F from stress-susceptible animal. 10μ thick sections, 64×.

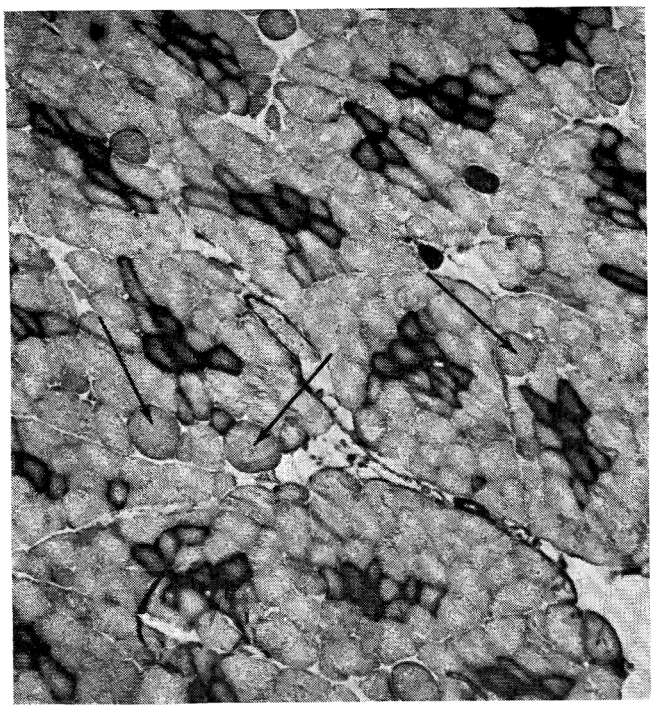

Fig. 3—Fresh frozen section of porcine longissimus muscle reacted for DPNH-TR. The typical morphology and distribution of giant fibers is shown. 10μ thick section, 44×.

fibers which are key contributors to the stress-susceptibility of the animal and to the development of PSE characteristics in the musculature.

Support was recently added to this hypothesis when muscle from a group of 20 animals of similar breeding and environmental conditions were examined. When the animals were transported only a short distance from holding facility to abattoir, eight died because they were acutely stress-susceptible. Histochemical study of the muscle from the stress-susceptible animals revealed that the majority of the dark fibers were intermediate and reacted intensely for amylophosphorylase and ATPase. The stress-resistant animals had low to negative amylophosphorylase and ATPase activity in a high percentage of the dark fibers.

The second result of real significance from this group of animals was the finding of large numbers of giant fibers in muscles from all stress-susceptible animals, but none from stress-resistant animals. The frequency of occurrence of giant fibers is unknown. They apparently occur in PSE muscle, but not all PSE muscle contains them. The appearance of giant fibers is illustrated in Figure 3. The histochemical properties of giant fibers have been described elsewhere (Cassens et al. 1969b) and they have been implicated in the problem of stress-susceptibility and PSE muscle.

In conclusion, it seems that the response of a stress-susceptible animal to anoxia in its skeletal muscle is due to the large number of intermediate fibers which are dependent upon aerobic metabolism, but unlike typical red fibers they have especially high ATPase and phosphorylase activity, breaking down ATP and accelerating glycolysis to trigger a rapid glycolytic rate in the entire muscle. Additionally, even the regular white, and to a lesser extent the regular red fibers have rather intense ATPase and phosphorylase activity and further contribute to the acceleration of these metabolic phenomena in the muscles of stress-susceptible animals. These phenomena are occurring in vivo during stress periods (Sair et al., 1969) and post-mortem after succumbing during the stress period or with exsanguination. In any of these cases, PSE characteristics develop in the skeletal musculature, and are most probably related to the fiber characteristics.

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Turbidity, Viscosity and ATPase Activity of Fibrillar Protein Extracts of Rabbit Muscle

SUMMARY——Results suggest that actomyosin from muscle in rigor is more easily dissociated than actomyosin from prerigor muscle. The addition of $MgCl_2$ decreased the rate of ATP hydrolysis and extended the clear phase of the fibrillar protein extract. Pyrophosphate in the presence of magnesium was as effective as ATP in clearing actomyosin. As the ATP was hydrolyzed, the viscosity of the solution returned to values greater than those obtained before the addition of ATP. However, samples cleared with pyrophosphate retained their low viscosity characteristics indefinitely.

INTRODUCTION

IMMEDIATELY following slaughter, muscle proteins possess excellent hydration and fat emulsifying properties, and the meat is generally considered very tender. During the development of rigor mortis, certain biochemical and physical changes occur that result in a decrease of muscle hydration and fat emulsifying properties as well as in the tenderness of the meat.

Fujimaki et al. (1965) showed that extracts of myosin B type protein from rigor muscle showed relatively higher values for ATPase activity, ATP sensitivity and reduced viscosity than myosin B prepared from pre-rigor muscle. Robson et al. (1966) also reported that the specific activity of myosin B prepared from rigor muscle stored for 12–24 hr postmortem was higher than that for myosin B prepared immediately after death. The present study was conducted in an effort to determine if there is any difference in the turbidity, viscosity and ATPase activity of a fibrillar protein extract from prerigor and rigor muscle. The effect of ATP and pyrophosphate on the viscosity of the myosin components of the fibrillar protein extract was also compared.

EXPERIMENTAL

EIGHT RABBITS weighing between 1.5 and 3.0 kg were used. A fibrillar protein extract was prepared from the longissimus dorsi muscle from the right side of the rabbit immediately after bleeding. After the remainder of the carcass had been chilled 24 hr at 0°C, a fibrillar protein extract was prepared from the longissimus dorsi muscle from the left side by the same procedure. The properties of the fibrillar protein extracts were investigated by measuring the ATPase activity and the changes in turbidity and viscosity.

Preparation of fibrillar protein extracts

The fibrillar protein extract of the longissimus dorsi muscle of the rabbit was prepared according to the method of Mihalyi et al. (1966). The muscle was dissected free of fat and connective tissue and homogenized in a Waring blender with four volumes of Weber-Edsall solution for 30 sec. The suspension was poured into a 250 ml centrifuge tube and left for 24 hr. At the end of this time, two volumes of Weber-Edsall solution were added and the solution was stirred thoroughly before centrifuging at 2,600 rpm for 20 min.

The myosin and actomyosin components of the supernatant were precipitated by diluting with 10 volumes of deionized water to remove the water-soluble muscle constituents. The precipitate was collected after centrifugation for 20 min at 2,600 rpm and was dissolved in 0.6*M* KCl. The myosin and actomyosin components, which were dissolved in 0.6*M* KCl, were clarified by centrifugation for 10 min at 12,000 rpm. The supernatant was then diluted to approximately 0.2% protein with 0.6*M* KCl. All extraction steps were carried out at 0–3°C. All nitrogen analyses were performed by the micro-Kjeldahl method, and the final protein concentration was calculated assuming that the fibrillar extract contained 16.15% nitrogen (Mihalyi et al., 1966).

ATPase activity and changes in turbidity

For measuring changes in turbidity and ATPase activity, the purified myosin and actomyosin components were diluted with 0.6*M* KCl to a final concentration of 0.675 mg of protein per ml. For determining the enzymatic activity and the changes in turbidity, 1 ml 0.01*M* $MgCl_2$ was added to 23 ml of the diluted protein extract. The reaction was started by adding 1 ml of a 0.01M ATP solution (buffered at pH 6.4 with Tris buffer) to 24 ml of the $MgCl_2$-protein solution. This was mixed immediately, poured into a cuvette and changes in turbidity were measured at 350 mμ (Perry et al., 1965) with a Beckman DU spectrophotometer equipped with a Gilford, Model 220, absorbence indicator. The reference turbidity was taken from a solution containing 0.5 ml of 0.01*M* $MgCl_2$, 0.5 ml deionized water and 11.5 ml of the diluted muscle extract. Changes in turbidity and ATPase activity were also measured by adding 1 ml of the 0.01*M* ATP solution to 24 ml of the protein solution without adding $MgCl_2$.

ATP was determined on 1 ml aliquots of the reaction mixture removed at appropriate times. The reaction mixture was immediately pipetted into boiling water to stop all enzymatic activity. The concentration of ATP was determined by the bioluminescence method described by Strehler et al. (1952) and Strehler (1953) using an Aminco-Bowman spectrophotofluorometer.

Viscosity

The viscosity of a 0.6*M* KCl solution of the purified Weber-Edsall extract was estimated by an Ostwald viscometer at pH 6.4. The relative change in viscosity of the protein solution was measured following the addition of 1 ml of 0.1*M* potassium pyrophosphate to a solution containing 1 ml of 0.01*M* $MgCl_2$ in 23 ml of the protein extract. The change in viscosity following the addition of ATP was measured in a similar manner.

RESULTS & DISCUSSION

ATPase activities and turbidity

Following the addition of ATP to the fibrillar protein, only 20–25% of the added ATP was detected after 1 min using the bioluminescence assay. The great initial drop in free ATP could be the result of binding of ATP to the fibrillar protein as well as to the high initial ATPase activity. As shown in Figure 1, the rapid reduction in free ATP was accompanied by an almost instantaneous decrease in the turbidity of the solution. After the initial decrease in turbidity, it remained relatively constant until the level of ATP decreased below 15%, at which time the turbidity increased.

The addition of ATP causes dissociation of the actin-myosin complex (Fig. 1), as shown by a decrease in absorbence. In the absence of adding Mg^{++}, ATP is hydrolyzed quite rapidly. As the ATP level falls below $10^{-6}M$ (between 10–30 min), it fails to keep the actin-myosin

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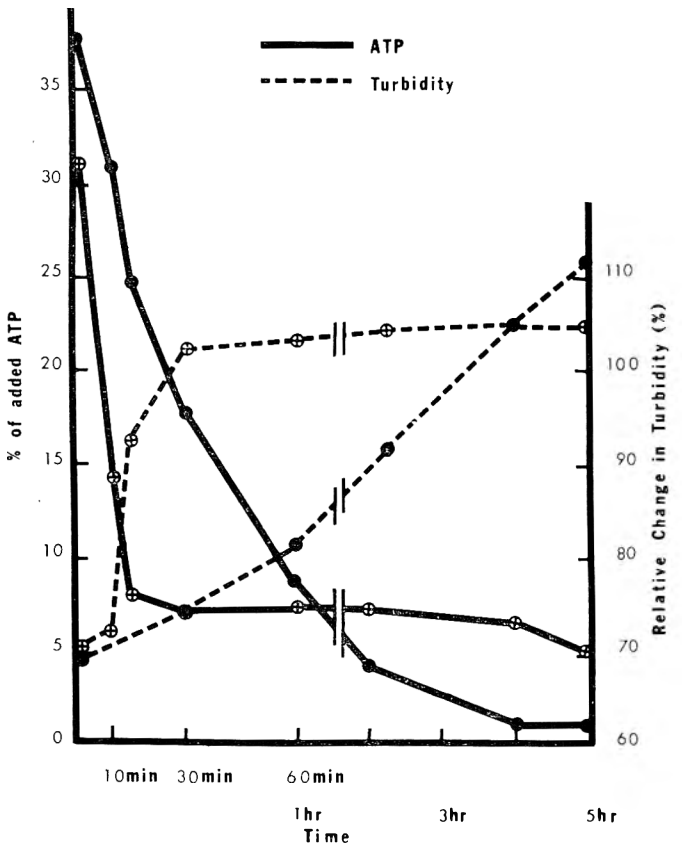

Fig. 1—Effect of $MgCl_2$ on the ATPase activity and relative changes in turbidity of pre-rigor fibrillar protein extract at 25°C. (⊕) The reaction was started by adding 1 ml of 0.01M ATP (pH 6.4) to 24 ml of the diluted protein extract. (●) One ml of 0.01M $MgCl_2$ was added to 23 ml of the diluted protein extract and the reaction was started by adding 0.01M ATP.

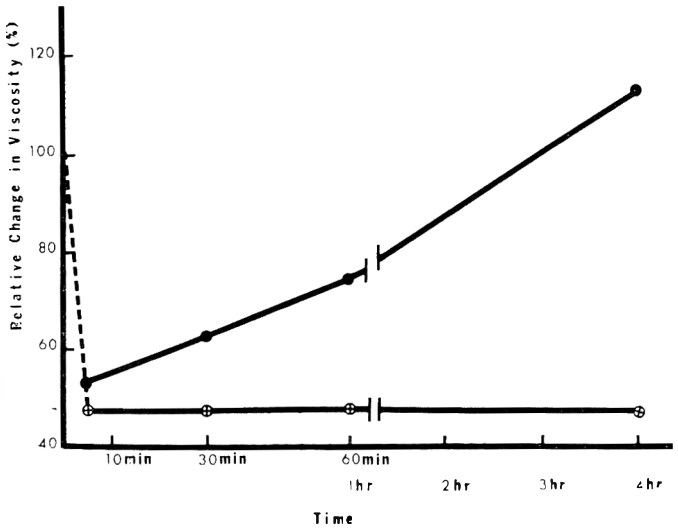

Fig. 2—The relative changes in viscosity of fibrillar protein extracts from muscle in rigor. One ml of 0.1M potassium pyrophosphate or 0.01M ATP (pH 6.4) was added to 24 ml of fibrillar protein extract containing approximately 0.2% protein and 0.4 mM added $MgCl_2$. Changes in viscosity were recorded at 23° C for ATP (●) and at 3°C for potassium pyrophosphate (⊕).

complex dissociated and a recombination occurs as shown by an increase in absorbency. Since added Mg^{++} not only inhibits ATPase activity but also assists ATP in keeping the actin-myosin complex dissociated, the entire process is much slower in the presence of added Mg^{++}.

In Figure 1, it can be seen that the addition of $MgCl_2$ suppressed the ATPase activity of the fibrillar protein extract and extended the period of time during which the actomyosin was dissociated (clear phase). Kaldor et al. (1963) and Goll et al. (1967) also found that magnesium inhibited the myofibrillar ATPase activity if 0.3 to 0.5*M* KCl was incorporated into the buffer. Present results agree with those of Noda et al. (1958), who showed that the addition of magnesium ions to a cleared actomyosin solution extended the time period for the clear phase.

Maruyama et al. (1962) and Ikemoto et al. (1966) suggested that the low ATPase activity of the actomyosin solution in the presence of magnesium ions, as found in the present experiment, is attributable to the magnesium inhibited ATPase activity of the myosin moiety. These results give direct evidence that the actin-myosin complex is dissociated as recently described by Goll et al. (1967).

The data in Table 1, show that after 5 hr the concentration of ATP in the absence of $MgCl_2$ was about five times as high as the level in the presence of $MgCl_2$. Statistical analysis revealed that these differences were significant at the 0.05 level. Results of Levy et al. (1966) indicated that magnesium and ATP are bound to the hydrolytic site on myosin. Szent-Györgyi (1951) stated that magnesium has a high affinity for myosin, if present in high concentrations, and that bound magnesium ions result in the absorption of ATP to the myosin molecule. Therefore, the more complete hydrolysis of ATP in the presence of $MgCl_2$ may be the result of increased binding of ATP to the hydrolytic site.

The ATPase activity and relative change in turbidity for the different extraction times are shown in Tables 1 and 2, respectively. Except for the significantly lower turbidity values at 1 and 15 min for samples extracted 24 hr post mortem, no differences were observed in ATPase activity and turbidity between the fibrillar protein extracts isolated from prerigor muscle and muscle in rigor. Results suggest that actomyosin extracted from muscle in rigor is indeed more easily dissociated than actomyosin extracted from pre-

Table 1—Effect of $MgCl_2$ on the ATPase activity of the fibrillar protein extracts at 25°C.

Post-mortem time of extraction	Added $MgCl_2$	Time after adding ATP[2] 1 min	10 min	15 min	20 min	1 hr	2 hr	5 hr
0 hr	Yes	37.8	31.0	24.8	17.6	8.87	4.2	1.2
0 hr	No	31.2	14.6	8.1	7.0	7.1	7.5	6.1
24 hr	Yes	39.8	33.0	26.8	18.4	8.5	4.8	1.5
24 hr	No	32.6	22.5	11.8	9.1	10.1	7.4	6.2

[1] The reaction was started by adding 1 ml of 0.01*M* ATP (pH 6.4) to 24 ml of the diluted protein extract. When $MgCl_2$ was added, 1 ml of 0.01*M* $MgCl_2$ was added to 23 ml of the diluted protein extract and the reaction was started by adding ATP.

[2] Values are expressed as % of added ATP.

Table 2—Effect of $MgCl_2$ on the relative change in turbidity of the fibrillar protein extracts at 25°C following the addition of ATP.[1]

Post-mortem time of extraction	Added $MgCl_2$	Time after adding ATP[2] 1 min	15 min	30 min	1 hr	2 hr
0 hr	Yes	68.9	72.6	74.3	81.8	91.5
0 hr	No	71.0	92.7	102.6	103.6	104.8
24 hr	Yes	62.8	69.6	74.3	81.2	91.9
24 hr	No	63.9	73.0	102.9	105.0	106.9

[1] The reaction was started by adding 1 ml of 0.01*M* ATP (pH 6.4) to 24 ml of the diluted protein extract. When $MgCl_2$ was added, 1 ml of 0.01*M* $MgCl_2$ was added to 23 ml of the diluted protein extract and the reaction was started by adding ATP.

[2] Reference optical density values for turbidity ranged from 0.081 to 0.354. Values are expressed as relative change in turbidity in regard to the reference solution.

Table 3—The relative changes in viscosity of the fibrillar protein extracts from prerigor and rigor muscle following the addition of pyrophosphate.[1]

Postmortem time of extraction	Time 5 min	1 hr	5 hr
0 hr	47.0	47.0	47.0
24 hr	47.9	46.4	47.0

[1] The changes in viscosity were recorded at 3°C following the addition of 1 ml of 0.1*M* potassium pyrophosphate to 24 ml of the fibrillar protein extract containing approximately 0.2% protein and 0.4-m*M* added $MgCl_2$.

rigor muscle. Fujimaki et al. (1965) and Robson et al. (1966) have reported that the specific activity of myosin B prepared from rigor muscle was higher than that for myosin B prepared immediately after death.

Viscosity

The data in Table 3 indicates very little difference in the relative changes in viscosity of the fibrillar protein extracts from the prerigor and rigor muscle following the addition of pyrophosphate. Thus, the interaction of actin and myosin in both pre- and postrigor muscle was weak enough that it was completely dissociated by pyrophosphate in 0.55*M* KCl.

A number of investigators (Noda et al., 1958; Watanabe et al., 1960; Tonomura et al., 1961; Yasui et al., 1964; Mihalyi et al., 1966) have shown that pyrophosphate in the presence of magnesium is as effective as ATP in dissociating actomyosin. Results from the present experiment (Fig. 2) also indicate that pyrophosphate in the presence of magnesium is as effective as ATP in clearing actomyosin. As the ATP was hydrolyzed, however, the viscosity of the solution returned to values greater than those obtained before the addition of ATP. On the other hand, samples cleared with pyrophosphate in the presence of magnesium retained low viscosity characteristics indefinitely.

The results of Bendall (1954) and Hellendoorn (1962) showed that low concentrations of pyrophosphate in combination with NaCl increase the water-binding of cooked meat. Immediately after slaughter, muscle is usually in a relaxed state, has a high level of ATP and has excellent hydration and fat emulsifying properties. As the level of ATP declines, actin and myosin complex to form actomyosin with a decrease in the hydration and fat emulsifying properties of the meat. The results from this experiment suggest that the addition of ATP to the fibrillar protein extract dissociated actomyosin, but then when the ATP was hydrolyzed, the complex between actin and myosin was reformed. However, in the presence of pyrophosphate the actomyosin remained dissociated indefinitely. This indicates that pyrophosphate may increase the water-binding of cooked meat by retaining the dissociation of actomyosin, thus maintaining the dissociated condition of the muscle proteins similar to that found immediately after slaughter, when high levels of ATP are present.

The dissociating influence of the pyrophosphate system, as observed in the present study, could be conditioned by the Mg-pyrophosphate complex (Granicher-Frick, 1965). Martonosi et al. (1964) concluded that the binding site for the pyrophosphate on myosin is identical with a portion of the ATPase center of myosin. Results of the present experiment suggest that pyrophosphate may be bound to the site on myosin that is responsible for the splitting of the actomyosin complex, but it is not involved in the formation of a contractile complex of actin and myosin as apparently can occur in the presence of ATP.

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Connective Tissues from Normal and Pale, Soft and Exudative (PSE) Porcine Muscles. 2. Physical Characterization

SUMMARY—*The epimysial connective tissues from normal and PSE longissimus dorsi muscles were subjected to physical characterization. No differences in conventionally determined shrinkage temperatures were observed. However, differential thermal analysis (DTA) studies revealed that epimysium from PSE tissues had lower onset and recovery temperatures and contained a higher percentage of components melting at low temperatures than that from normal tissues. The epimysium from PSE muscles also had a higher initial moisture and lower dry matter content. Epimysial connective tissues underwent osmotic swelling in neutral solutions, with those from PSE muscles imbibing significantly more water than that from normal muscles. Dry matter content decreased during neutral swelling, probably due to a loss of ground substance. Collagen from PSE muscles had a molecular weight between cross-links (Mc) of 6.37 × 10⁴ and a resultant cross-link density of 5.23/molecule, while normal tissues had corresponding values of 4.67 × 10⁴ and 7.73. These differences were only significant at the 10% level.*

INTRODUCTION

SEVERAL WORKERS have indicated that the connective tissues may be altered in muscles from pigs exhibiting the pale, soft and exudative (PSE) condition (Sayre et al., 1963; Briskey, 1964). Very little emphasis has, however, been placed on the nature of these alterations. McClain et al. (1968a) reported an altered or decreased ground substance content in the epimysium from PSE longissimus dorsi muscles. A subsequent report (McClain et al., 1969a) revealed that epimysium from PSE tissues had a higher content of newly synthesized tropocollagen. In addition, the collagen proteins had lower numbers or decreased strength of cross-links in comparison to normal tissue.

This study was initiated to further characterize the chemical and physical nature of the epimysial connective tissues associated with porcine muscles developing the PSE condition.

MATERIALS & METHODS

Shrinkage temperature (Ts)

The epimysium from six PSE and six normal l. dorsi muscles from market weight carcasses was removed and handled as described previously McClain et al., 1968a). Thermal shrinkage temperatures were determined on epimysial collagen by a method similar to that of Gross (1964). Samples were floated in 0.9% sodium chloride on the heating stage of a Fisher-Johns melting point apparatus. A cover slip was placed over the heating stage, and the samples were subjected to heat at the rate of 3°C/min. The temperature was noted and recorded at the beginning of shrinkage, at the end of shrinkage and at the temperature of complete dissolution.

Differential thermal analysis (DTA)

The instrument used for this investigation was the Du Pont 900 Differential Thermal Analyzer. The technique utilized has been reported previously (McClain et al., 1969b). Approximately 30 mg of sample was used, and the heating rate was 10°C/min under an air atmosphere. The T scale was set at 10°C/min, the ΔT scale at 0.2°C/in. and the base line slope at −0.2.

Stress-strain and swelling measurements

The stress-strain method used was essentially that of Wiederhorn et al. (1953). The apparatus utilized has been described previously by McClain et al. (1969b). Swelling characteristics of the epimysial connective tissues were evaluated by the change in weight observed after soaking in various solvents. Dimensional changes were also measured for the swollen samples.

RESULTS & DISCUSSION

Thermal shrinkage

Temperatures at the onset of shrinkage, completion of shrinkage and denaturation were very similar in epimysium from both PSE and normal tissues (Table 1). The differences between the various temperature parameters were not statistically significant. These results were surprising in light of previously reported results (McClain et al., 1969a) which indicated a decrease in the amount of cross-linking, or a less stable type collagen in the epimysium from PSE muscles.

Differential thermal analysis

Results of the DTA study on epimysium from normal and PSE tissues are shown in Table 2. Although differences between the epimysium from normal and PSE muscles appeared to be small, analysis of variance revealed they were highly significant ($P < 0.01$) in all four DTA parameters.

On the basis of the data in Table 3, it is apparent that epimysium from PSE muscles had a total melting point range similar to that for normal muscles. However, onset and recovery occurred at lower temperatures. The PSE tissues also contained a higher percentage of components or cross-links that melted at a low temperature. This probably explains the increased amount of salt-soluble collagen and the greater release of heat labile collagen reported for the epimysium from PSE tissues (McClain et al., 1968a).

The results of the DTA study also explain why no differences in Ts values were observed between normal and PSE epimysium in the thermal shrinkage studies conducted with the melting point apparatus. It is readily apparent that thermal

Table 1—Thermal shrinkage temperature (Ts, °C) of epimysial connective tissues from normal and PSE l. dorsi muscles.

PSE				Normal			
Animal no.	Onset of shrinkage[1]	Completion of shrinkage[2]	Denaturation[3]	Animal no.	Onset of shrinkage[1]	Completion of shrinkage[2]	Denaturation[3]
1	60.00	61.00	64.00	2	59.00	60.00	62.00
4	58.50	59.50	61.00	3	59.00	60.00	62.00
6	59.00	60.00	61.00	5	59.00	60.00	61.50
Mean	59.16	60.16	62.00		59.00	60.00	61.83

[1] Temperature at beginning of shrinkage.
[2] Temperature at end of shrinkage.
[3] Temperature at complete loss of opacity.

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Table 2—Differential thermal analysis of epimysial connective tissues from normal and PSE l. dorsi muscles.

Animal no.	Onset,[1] °C	Extrapolated[2] onset, °C	Peak,[3] °C	Recovery,[4] °C
PSE				
1	46.41	58.88	65.87	78.99
4	46.98	59.47	65.97	78.99
6	46.25	59.00	65.64	76.03
10	49.69	58.62	65.25	74.94
8	46.04	58.58	65.41	76.60
14	45.42	57.96	65.87	78.37
Mean	46.79[5]	58.75[5]	65.67[5]	77.32[5]
Std. dev.	1.50	0.50	0.29	1.70
Normal				
2	49.38	60.93	66.81	81.25
3	48.75	59.63	66.03	80.98
5	49.37	59.15	66.03	77.96
7	50.00	60.35	66.70	81.48
11	49.74	60.18	66.03	81.19
13	49.37	60.45	66.19	82.93
Mean	49.44	60.12	66.30	80.97
Std. dev.	0.42	0.63	0.36	1.63

[1] Onset = temperature at which thermogram departs from baseline. It is the first indication of a physical change (McClain et al., 1968b).

[2] Extrapolated onset = temperature corresponding to the intersection of extrapolations of the baseline and the longest straight line section on the low temperature side of peak (McClain et al., 1968b). It represents the starting temperature of the major portion of the transformation.

[3] Peak = temperature of reversal or the temperature at which the differential between the sample and reference is greatest. In compounds undergoing fusion, this represents the melting point (McClain et al., 1968b).

[4] Recovery = temperature at which the thermogram returns to either the same or a different baseline (McClain et al., 1968b).

[5] Difference between means for epimysium from PSE and normal muscles significant at $P < 0.01$ level.

Table 3—DTA[1] data on melting ranges for epimysial connective tissues from normal and PSE l. dorsi muscles.

	Source of epimysial tissues[2]	
	Normal muscles	PSE muscles
Total melting range—°C	31.53	30.53
Onset to peak—°C	16.86[3]	18.88
Onset to peak—% of total melting range	53.50[3]	61.80
Peak of recovery—°C	14.67[3]	11.65
Peak of recovery as % of total melting range	46.50[3]	38.20

[1] DTA—Differential thermal analysis.

[2] All muscles from animals approximately 5 months old.

[3] Difference between means for epimysium from PSE and normal muscles significant at $P < 0.01$ level.

shrinkage does not occur at a given temperature, but rather over a wide and variable range. In addition, the differences in DTA peak temperature were very slight, although significant at the 1% level, and probably would not be detected by conventional methods.

It is interesting to note that the DTA parameters for the epimysium from PSE muscles are intermediate to those for the 7 week-old pigs as reported by McClain et al. (1968b), and the normal 5 month-old pigs from the present study. The workers cited above reported a total melting point range from onset to peak and from peak to recovery, respectively, of 62.40 and 37.60% for the epimysium from 7 week-old pigs. These parameters were 61.80 and 38.20% for the epimysium from 5 month-old pigs exhibiting the PSE condition, and 53.50 and 46.50% for epimysium from normal 5 month-old pigs in the present study.

Swelling and stress-strain studies

The results of a swelling study conducted on epimysial connective tissues from normal and PSE muscles are shown in Table 4. Analysis of variance revealed a significantly ($P < 0.05$) lower initial dry matter content and a higher initial moisture content in the epimysium from PSE tissues. These differences approached significance at the 1% level. No significant differences were evident between the two tissue types in the dry matter content after soaking. The epimysium from PSE tissues lost an average of 4.90% dry matter during soaking, while the normal tissue lost 6.49%.

The lower dry matter content and decreased loss of dry matter from the PSE tissues during soaking in water could reflect a lower ground substance content. This conclusion is substantiated by the findings of McClain et al. (1968a), which revealed a lower content of metachromatic staining material in the epimysium from PSE pigs.

There was also a significantly greater ($P < 0.05$) amount of water imbibed by epimysium from the PSE pigs. The increased water uptake in epimysium from PSE tissues is not completely compatible with our knowledge of water-binding by connective tissues. Ground substance is noted for its water holding capacity. Thus, the apparent low content of ground substance in the epimysium from PSE tissues should limit the water imbibing capacity of the tissues. On the other hand, if the

Table 4—Neutral swelling characteristics of epimysial connective tissues from normal and PSE l. dorsi muscles.

Animal no.	Initial moisture,[1] %	Initial dry matter,[1] %	Dry matter after soaking,[1] %	Dry matter lost,[1] %	Water uptake,[2] %
PSE					
1	63.25	36.75	29.28	7.47	10.35
4	62.74	37.26	33.76	3.50	11.00
6	61.90	38.10	36.75	1.32	14.78
8	63.78	36.22	28.92	7.30	10.47
10	63.01	36.99	31.88	5.11	13.84
14	63.94	36.06	31.88	4.68	16.58
Mean	62.96[3]	36.90[3]	32.08	4.90	12.84[3]
Std. dev.	0.62	0.74	2.92	2.33	2.61
Normal					
2	59.84	40.16	34.46	5.70	5.41
3	62.14	37.86	35.78	2.08	10.29
5	58.96	41.04	36.65	5.39	12.85
7	56.50	43.50	29.69	13.81	9.03
11	61.33	38.67	29.49	9.18	7.27
13	62.17	37.83	35.05	2.78	4.63
Mean	60.16	39.84	33.35	6.49	8.25
Std. dev.	2.20	2.20	2.95	4.38	3.10

[1] Percent of initial wet weight of tissue.

[2] g water/g tissue.

[3] Differences between means for epimysial connective tissues from PSE and normal muscles significant at $P < 0.05$ level.

Table 5—Results of stress-strain measurements on epimysial connective tissues from normal and PSE l. dorsi muscles.

PSE			Normal		
Animal no.	Mc[1] × 10^4	Cross-links/ molecule[2]	Animal no.	Mc[1] × 10^4	Cross-links/ molecule[2]
14	4.34	6.90	7	2.97	10.09
4	10.83	2.76	2	9.10	3.30
1	7.65	3.91	13	5.91	5.08
6	4.90	6.11	3	2.51	11.95
8	4.50	6.68	11	3.60	8.33
10	5.94	5.04	5	3.92	7.64
Mean	6.37	5.23		4.67	7.73
Std. dev.	2.51	1.65		2.46	3.17

[1] Mc = molecular weight between cross-links.
[2] Assuming a molecular weight of 300,000 for the collagen molecule.

swelling characteristics of collagen are reflections of looser molecular ordering and less cross-linking (Gustavson, 1956; Fels, 1966), then the epimysium from PSE tissues would be expected to imbibe larger amounts of water.

The results of stress-strain studies on epimysial connective tissues from normal and PSE muscles are shown in Table 5. The molecular weight between covalent cross-links (Mc) was 6.37 × 10^4 in the case of the epimysium from PSE tissues and 4.67 × 10^4 for the normal tissues. The corresponding cross-linking values were 5.23 and 7.73, respectively. These differences were, however, only significant at the 10% level. Here again, it is interesting to note that stress-strain values found for the epimysium from PSE pigs were very similar to those of the 7 week-old baby pig reported by McClain et al. (1968a).

Results of this and previous studies (McClain et al., 1969a,b) show that connective tissue from PSE pigs is altered as compared to that from normal animals. The epimysium from PSE muscles apparently has a lower or altered ground substance content and the collagenous proteins are less mature, containing fewer intra- and probably inter-molecular cross-links. Whether the altered connective tissue make-up is actually a causative factor in the development of the PSE condition or merely a result of rapid post-mortem changes remains to be elucidated.

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Studies on Natural Actomyosin: Survey of Experimental Conditions

SUMMARY—Natural actomyosin was prepared from the longissimus dorsi of both bovine and rabbit. Rabbit actomyosin had about two times the ATPase activity of bovine muscle actomyosin. However, both types of natural actomyosin behaved in a similar manner with respect to ionic conditions. They were activated by Mg^{++} at low KCl (20–50 mM), inhibited by Mg^{++} at high KCl (100 mM) and activated by Ca^{++} at high KCl (100 mM). Bovine actomyosin, in contrast to rabbit actomyosin, did not show activation at low Mg^{++} (0.01 mM) in the alkaline pH range. Maleate, when used as Tris-Maleate buffer, inhibited superprecipitation of natural actomyosin from rabbit and bovine muscle. It was hypothesized that the low ATPase activity and the non-activation at low Mg^{++} in the alkaline pH range of bovine compared to rabbit actomyosin was a species difference.

INTRODUCTION

THE INFLUENCE of various ions and ionic concentrations on the ATPase activity of natural actomyosin has been well documented (Weber, 1959; Nihei et al., 1966; Watanabe et al., 1964, 1965). Various electrolytes, especially the alkaline earth metals, Ca^{++} and Mg^{++}, have been shown to alter the reactions and interactions of actomyosin and ATP. At sufficiently high ionic strength, addition of ATP and Mg^{++} results in clearing of actomyosin (Spicer, 1952; Maruyama et al., 1962a,b). On the other hand, at low ionic strength ATP and Mg^{++} cause a rapid onset of superprecipitation (Szent-Györgyi, 1947; Ebashi, 1961). Calcium

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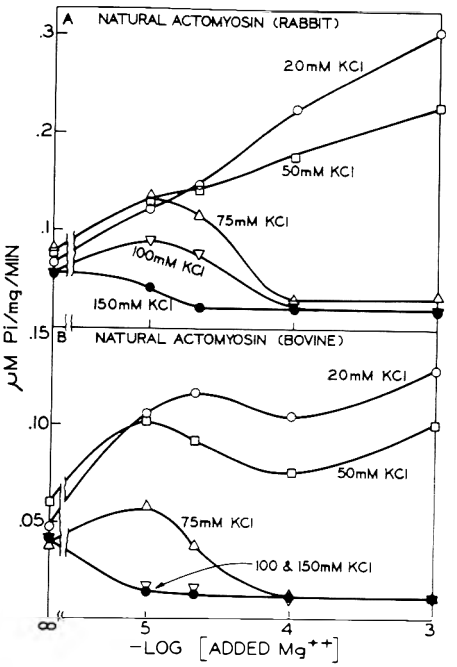

Fig. 1—Effect of KCl and Mg^{++} concentrations on ATPase activity of actomyosin. Final concentration: KCl and Mg^{++} as indicated; 1 mM ATP; 20 mM tris-acetate (pH 6.8); 0.33 mg actomyosin/ml; [Mg^{++}] in M. Temp. 25°C.

by itself stimulates actomyosin ATPase; however, the degree of superprecipitation is in many cases either antagonized or depressed by Ca^{++} (Szent-Györgyi, 1943; Azzone et al., 1964; Watanabe et al., 1965). Nevertheless, Ca^{++}, in the presence of ATP and Mg^{++}, is thought to be the key cation that regulates or controls activity of natural actomyosin (Ebashi, 1961; Weber et al., 1964).

The purpose of the experiment was to establish the conditions under which the properties of bovine actomyosin could be studied with the ultimate objective of relating such properties to tenderness. The properties of rabbit actomyosin under various conditions are well known. Therefore, we used rabbit actomyosin as a standard of comparison in the investigation of bovine actomyosin. The influence of ionic strength, various cations, EDTA, pH and buffer composition on the properties of bovine and rabbit actomyosin are described in this paper.

EXPERIMENTAL

Preparation of natural actomyosin

Natural actomyosin was prepared from the longissimus dorsi of rabbit and bovine animals according to the procedure of Endo (1964), with slight modifications. Minced muscle (50 g) was mixed with three volumes of water, homogenized for 3 min in a Waring Blendor, and centrifuged 10 min at 7,000 × *g* to remove sarcoplasm. The residue was suspended in four volumes of 0.6 M KCl with 0.01 M $NaHCO_3$. After a 6–8 hr extraction period with slow gentle stirring at approximately 50 rpm, the solution was centrifuged at 15,000 × *g* for 1 hr and the residue was discarded. Two volumes of cold water were added to the supernatant, stirred gently to precipitate the actomyosin and centrifuged 10 min at 7,000 × *g*. The residue was dissolved in 3 M KCl to a final concentration of 0.6 M, and then 0.15 M KCl was added with gentle stirring to bring the KCl concentration to 0.25 M. The solution was then centrifuged for 10 min at 7,000 × *g*. The residue was dissolved in 3 M KCl to a final KCl concentration of 0.6 M. Cold water was then added with gentle stirring to a KCl concentration of 0.15 M. The solution was centrifuged 10 min at 4,000 × *g*, decanted and recentrifuged 10 min at 7,000 × *g*. The precipitated actomyosin was dissolved in 1 M KCl to bring the concentration to 0.6 M, after which it was clarified at 20,000 × *g* for 1 hr. All operations were performed below 4°C and, unless noted otherwise, all solutions also contained 0.5 mM $NaHCO_3$. Samples were stored in a deep freeze at −20°C with an equal volume of glycerol at least 1 week before use. Samples used in experiments were washed free of glycerol by adding water to a concentration of 50 mM KCl and washed twice more with 50 mM KCl. They were dissolved in 0.6 M KCl and clarified at 20,000 × *g* for 1 hr. Such preparations were held at 0°C and used within 10 days.

Measurement of ATPase activity

Actomyosin ATPase activity was determined by measuring inorganic phosphate liberation at 25°C. The actomyosin was suspended in the appropriate reaction medium (see table and figures) and 30 sec later the reaction was started by adding ATP. Aliquots for phosphate analysis were removed at regular intervals and the reaction was terminated by addition to cold 15% TCA. Inorganic phosphate was determined according to the method of Fiske et al. (1925), as modified by Ouass et al. (1968), at 37°C, with a reaction time of exactly 10 min. ATP hydrolysis was followed and was negligible during this period of color development. The initial reaction rate was established by plotting mM phosphate concentration versus time, and the slope of the line was determined. Results were expressed as μM P_i/mg protein per min.

Superprecipitation of actomyosin

The turbidity method of Seraydarian et al. (1967) was used, except a Beckman D.B. Spectrophotometer with a Texas Instrument recorder was employed (at 660 m). A protein concentration of 0.35 mg/ml was used and the actomyosin was suspended in the electrolyte medium, with KCl added first and Mg^{++} added just prior to ATP addition. All reactions were conducted at 25°C.

Protein concentration

Protein concentrations were measured by the biuret method (Gornall et al., 1949) standardized against crystalline bovine serum albumin.

Materials

All reagents were analytical reagent grade. The disodium salt of ATP (Sigma Chemical Co., St. Louis, Mo.) was washed in Dowex 50 and neutralized with KOH before use.

RESULTS & DISCUSSION

Effect of KCl and Mg^{++} concentrations on ATPase activity

Both rabbit and bovine actomyosin were similar in the response of ATPase activity to increased KCl and Mg^{++} concentrations (Fig. 1). The activity of rabbit natural actomyosin (Fig. 1a) was about twice that of bovine natural actomyosin (Fig. 1b). At low (20 mM) KCl concentration, increasing the Mg^{++} concentration resulted in the increased activation of the ATPase activity. When the KCl concentration was increased, the activity-Mg^{++} relationship shifted to bell-shaped curves emphasizing the diphasic response to increasing Mg^{++} (Watanabe et al., 1964). At high KCl concentration, Mg^{++} exerted an inhibitory effect, especially for bovine actomyosin, even at 10^{-5} M Mg^{++} as the clearing reaction was observed concomitantly with the slow ATPase activity. No attempts were made to remove Ca^{++} from reagent grade KCl used in these experiments; therefore, the activating effect of Mg^{++} was easily demonstrated at low KCl concentrations. The inhibitory effect of high KCl concentrations on ATPase was due either to an increase in the ionic strength resulting in clearing (Maruyama et al., 1962a,b), or K^+ binding to protein (Maruyama et al., 1963; Nihei et al., 1966), or both. Nihei et al. (1966) have presented a simple mechanism whereby the binding of divalent cations to actomyosin explained the activation by Mg^{++} and an increase in K^+ concentration displaced divalent cations from binding sites resulting in inhibition. The clearing of actomyosin at an ionic strength of 0.15 to 0.16 brought about by ATP in the presence of Mg^{++} has been interpreted as being due to dissociation of actomyosin into its constituent proteins, myosin and actin (Maruyama et al., 1962a,b).

Interrelationships among KCl, Ca^{++}, and Mg^{++}

The effect of KCl concentration on Ca^{++}-activated ATPase is shown in Figure 2. Rabbit actomyosin behaved similarly to bovine actomyosin and optimum activity was reached in both at 100 mM KCl. The effect of increasing Ca^{++} in the presence of Mg^{++} is shown in Figure 3. Ca^{++} activated the ATPase of rabbit natural actomyosin at concentrations up to 1 mM; however, at 10 mM, Ca^{++} actually inhibited the ATPase. Bovine actomyosin at 75 mM KCl and 0.1 mM Mg^{++} did not respond to increasing Ca^{++} concentrations. This may have been due to displacement of protein bound Mg^{++} by Ca^{++} (Nihei et al., 1966). At high KCl (150 mM), Ca^{++}

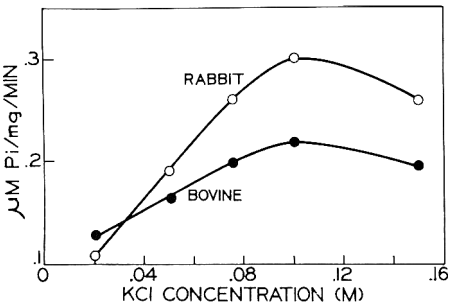

Fig. 2—Effect of KCl concentration on Ca^{++}-activated ATPase activity of natural actomyosin. Final concentration: 1 mM ATP; 20 mM tris-acetate (pH 6.8); 1 mM Ca^{++}; 0.33 mg actomyosin/ml. Temp. 25°C.

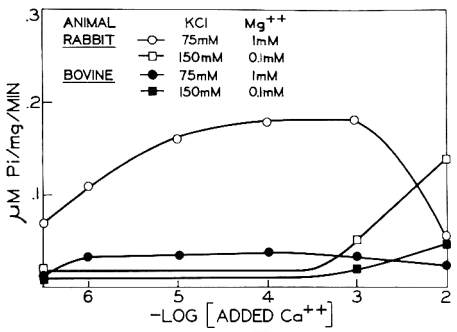

Fig. 3—Influence of Ca^{++}, Mg^{++}, and KCl on ATPase activity of natural actomyosin. Final concentrations: KCl and Mg^{++} as indicated; 1 mM ATP; 20 mM tris-acetate (pH 6.8); 0.33 mg actomyosin/ml; [Ca^{++}] in M. Temp. 25°C.

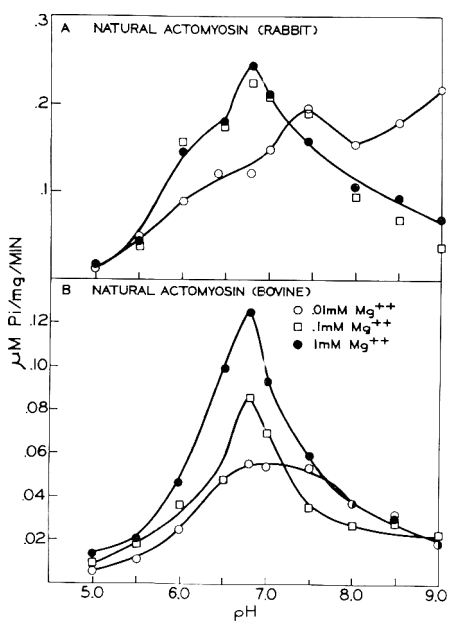

Fig. 4—Influence of pH on ATPase activity of natural actomyosin. Final concentrations: 50 mM KCl; 1 mM ATP; Mg^{++} as indicated; 20 mM tris-acetate; 0.33 mg/ml actomyosin. Temp. 25°C.

did not overcome inhibition by Mg^{++} until a concentration of 10 mM was reached. Barany et al. (1960) concluded that when Ca^{++} was the only bivalent cation present, actomyosin split ATP with a mechanism similar to that of myosin. Mühlrad et al. (1965) found there was an "essentiality of Ca^{++}" for ATPase of myofibrils and that free Ca^{++} at concentrations of 10^{-6} M suppressed the dissociation of actomyosin that occurred in the presence of sufficiently high Mg^{++} and ATP, but low free Ca^{++} (10^{-7} M). Weber et al. (1964) concluded that the Ca-actomyosin complex formed when the Ca^{++} concentration was sufficiently high reversed the dissociating effect of Mg^{++} in the presence of ATP.

Effect of EDTA

As shown in Table 1, EDTA washing of natural actomyosin had little effect on the activity of the Mg^{++}-activated ATPase (no attempt was made to remove Ca^{++} from the KCl used in these experiments). EDTA inhibited the ATPase of natural actomyosin in the presence of 1 mM Mg^{++} while addition of 1 mM Ca^{++} reversed the inhibition by EDTA and Mg^{++}. It is well established that Ca^{++} is needed for activity of natural actomyosin but not actomyosin reconstituted from pure myosin and pure actin (Perry et al., 1956; Weber et al., 1963; Ebashi et al., 1964). These studies implicated "native tropomyosin" (Ebashi, 1963) containing the Ca^{++}-sensitizing protein factor, troponin (Ebashi et al., 1965b), as being present in natural actomyosin, but not in reconstituted actomyosin. It appears, therefore, that bovine natural actomyosin also contains "native tropomyosin."

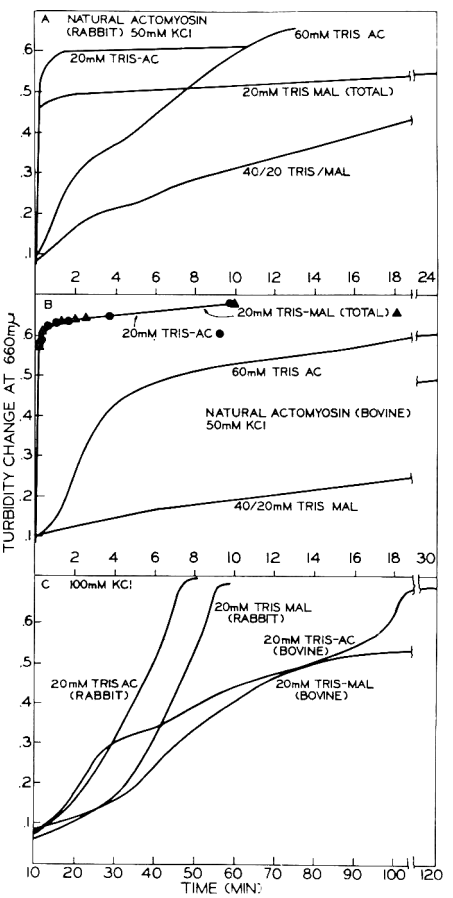

Fig. 5—Influence of buffer on superprecipitation of natural actomyosin. Final concentrations: KCl as indicated; 1 mM ATP; buffer as indicated (pH 6.8); 1 mM Mg^{++}; 0.35 mg/ml actomyosin. Temp. 25°C.

Effect of pH

Figure 4 summarizes the effect of pH on Mg^{++}-activated ATPase at various concentrations of Mg^{++}. The optimal pH for natural actomyosin of rabbit (Fig. 4a) and bovine (Fig. 4b) muscle was about pH 6.8 at 0.1 and 1 mM Mg^{++}. However, optimum activity was shifted to a higher pH for rabbit natural actomyosin (Fig. 4b) when 0.01 mM Mg^{++} was used. These data are in general agreement with those of Watanabe et al. (1965) and Carvalho et al. (1966) for ATPase of rabbit natural actomyosin, and of Maruyama (1966) for ATPase of insect actomyosin. That bovine actomyosin did not have its ATPase increase in a manner similar to rabbit actomyosin in the alkaline range (pH 7.5) at 0.01 mM Mg^{++} may be a reflection of a higher red fiber content in the bovine longissimus dorsi than in rabbit longissimus dorsi (Moody, 1967, personal communication). Barany et al. (1965) have shown the Ca^{++} and EDTA-activated ATPase of myosin from red muscle did

Table 1—Effect of EDTA on ATPase[1] of natural actomyosin

Treatment[2]	EDTA concentration (mM) 0	0.01	0.1	1.0
Rabbit				
Control[3]	.212	.168	.024	.018
EDTA-washed[4]	.220	.170	.034	.028
Control + 1 mM Ca^{++}	—	—	—	.231
EDTA-washed + 1 mM Ca^{++}	—	—	—	.242
Bovine				
Control[3]	.066	.054	.015	.011
EDTA-washed[4]	.064	.055	.014	.009
Control + 1 mM Ca^{++}	—	—	—	.056
EDTA-washed + 1 mM Ca^{++}	—	—	—	.076

[1] ATPase activity in μM P_i/mg per min.
[2] Final concentrations: 50 mM KCl, 20 mM tris-acetate, pH 6.8, 1 mM $MgCl_2$, 1 mM ATP. Temp. 25°C.
[3] Control samples were washed after removal from glycerol three times with 50 mM KCl.
[4] Samples were washed once after removal from glycerol with 50 mM KCl plus 1 mM EDTA and then twice more with 50 mM KCl.

not increase compared to myosin from white muscle in the alkaline pH range.

Superprecipitation and buffer composition

The influence of buffers on superprecipitation of actomyosin was investigated. Tris-maleate was used initially because Ebashi (1961) had used it in his studies of superprecipitation. However, we found that the rate of superprecipitation of bovine actomyosin was strongly inhibited by 40 mM tris–20 mM maleate (Fig. 5b). Tris-maleate also inhibited, but to a lesser degree, superprecipitation of rabbit natural actomyosin. When identical concentrations (60 mM) of trisacetate were used (Fig. 5) the inhibition of superprecipitation was less dramatic. These observations were interesting in view of the finding of Ebashi et al. (1965a) that α-actinin was necessary for superprecipitation to occur with reconstituted actomyosin. These investigators used tris-maleate in their reaction mixture. Only under very special conditions, including the presence of tris-maleate, could Briskey et al. (1967a,b) duplicate the findings of Ebashi et al. (1965a).

In searching for an explanation of the effect of the maleate ion it was found that maleate is classified as an enzyme and metabolic inhibitor (Webb, 1966). Webb suggested that maleate reacted with SH groups on proteins if steric conditions permitted, bound activating cations, chelated enzyme-bound metal ions, and competed with certain anionic substrates. The mechanism, therefore, whereby maleate could inhibit natural actomyosin is not known, but could possibly be by any of the mechanisms mentioned above, or by nonspecific ionic or ionic strength effects. This may be partially due to the double negative charge on the maleate molecule. Therefore, it was decided that further experiments with superprecipitation of natural actomyosin would be accomplished using tris-acetate instead of tris-maleate.

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Effect of Dietary Fats on Some Chemical and Functional Properties of Eggs

SUMMARY—Laying pullets were fed a low fat semipurified diet or the low fat diet supplement with 10% vegetable oil (corn, soybean, olive, safflower or hydrogenated coconut oil). The eggs were analyzed for change in the fatty acid composition of the total yolk lipids with time on the diet and for fatty acid composition of the triglyceride, cephalin and lecithin fractions of lipovitellin and lipovitellenin. Determinations were made for volume of sponge cakes, emulsification, and lipid content of stored eggs. A taste panel was used to assess any difference in flavor and mouth feel of yolk from stored eggs. The fatty acid composition of the total yolk lipids was influenced by all dietary fats. The major change was in the linoleic acid at the expense of oleic acid with corn, soybean and safflower oil. Olive oil increased the oleic acid and hydogenated coconut oil increased lauric, myristic and myristoleic acids. The fatty acid composition of the fractions of the lipoproteins was influenced by the dietary fats and varied between fractions. Differences were noted between sponge cake volume with eggs of low fat, corn oil, soybean oil and hydrogenated coconut oil diets. The dietary fats did not appear to affect emulsification capacity or migration of the yolk. A taste panel was unable to differentiate on the basis of flavor or mouth feel the egg yolk from the several treatments.

INTRODUCTION

THE CHANGES that occur in fatty acid composition of egg yolk lipids by modifying the dietary fat have been extensively studied since the early report of Cruickshank et al. (1939). Cruickshank et al. (1939), Reiser (1950), Fisher et al., (1957) and several others more recently have found the fatty acid composition of total yolk lipids to vary significantly, depending on the fatty acid composition of the dietary fats.

Most of the reports have dealt with modifying the unsaturated fatty acid composition of the total yolk lipids. Chung et al. (1964) reported increases in linoleic acid with comparable decreases in oleic acid when corn oil or soybean oil was included in the diet of the hens. They also found a large deposition of short-chain fatty acids (lauric, myristic and myristoleic acids) resulted from dietary supplementation with hydrogenated coconut oil.

There is relatively limited information on the effect of dietary fats on the glyceride and phospholipid fractions of the yolk (Reiser, 1950; Choudhury et al., 1959; Evans et al., 1961). Chen et al. (1965) reported that when linseed oil was included in the diet of the hen, linoleic acid was deposited to a greater extent in the triglyceride fraction than in the phospholipid fractions.

There is little information, however, upon the effect, if any, of different fats in the hen's feed upon the functional properties of the eggs produced. Jordan et al. (1962) reported a significant improvement in volume of sponge cakes made from eggs laid by hens fed a diet containing 10% corn oil. They found no effect of dietary fat on emulsifying capacity of egg yolk when comparing 10% corn oil or beef tallow to a low fat diet. Jordan et al. (1960) found a significant flavor difference between eggs from hens fed 10% beef tallow and a low fat ration.

This study was initiated to investigate the effect of additional dietary fats on the fatty acid composition of yolk lipids and the lipid fractions of the lipoproteins. The study also included the effect of changes in dietary lipids of the hens on some functional properties of the eggs.

EXPERIMENTAL PROCEDURES

Design of experiments

White Leghorn hens were randomly distributed into six groups of 30 hens each. The dietary treatments consisted of a low fat control ration (LF) (Table 1) and the low fat ration supplemented with 10% corn oil (CO), soybean oil (SO) hydrogenated coconut oil (HCO), safflower oil (SFO) or olive oil (OO). All groups were fed the control ration for two weeks; thereupon they were fed the respective experimental ration. After eight weeks on the experimental rations, they were again fed the control ration.

Rations containing the various vegetable oils were fed to hens to study influence of the oil on: (1) the total yolk lipid content, (2) the change in fatty acid composition of the total lipids of yolk with respect to time on the ration, and (3) the fatty acid composition of the triglyceride, lecithin and cephalin fractions of the lipovitellin and lipovitellenin.

The eggs were used to study the effect of the modified fatty acid composition on: (1) emulsification capacity of the whole yolk, (2) sponge cake volume, (3) flavor and mouthfeel of the yolk of stored eggs, and (4) migration of lipids from the yolk to the white during storage.

Egg yolk and white

Eggs were collected every Sunday, Tuesday and Thursday evening during the first three weeks and last three weeks of the experiment for analysis the following day. These analyses included emulsifying capacity of yolk, and sponge cake volume. The yolks from each treatment were pooled and blended in a Waring Blendor. The whites from all treatments were pooled, blended in a Waring Blendor and strained through cheese cloth. These whites were added back to the yolk in natural proportion for use in making sponge cakes.

Lipoproteins

Eggs were collected on Sunday of the fifth through eighth week and handled as indicated previously. Approximately 200 g of yolk from each treatment were used to prepare lipovitellin and lipovitellenin by the method of Evans et al. (1961).

Lipid extraction

Lipids were extracted from the whole yolk, by a modification of the method of Ostrander et al. (1961).

Table 1—Composition of low rat ration (%).

Ingredient	LF
Soybean oil meal (50%)	36.00
Cerelose	53.21
Vitamin premix[1]	1.00
$CaCO_3$ (ground)	6.25
Dicalcium phosphate	3.00
NaCl (iodized)	0.45
$MnSO_4$ (technical)	0.05
$ZnCO_3$	0.01
$FeSO_4 7H_2O$	0.02
Butylated hydroxy toluene	0.01

[1] Vitamin premix comprised of the following ingredients per 100 lb feed: Choline chloride (25%), 0.40 lb; Vitamin A (5000 USP/g), 0.20 lb; Vitamin D_3 (3000 USP/g), 0.05 lb; Vitamin mix 4-8-18 (Riboflavin, Ca pentothenate, Niacin), 0.10 lb; Thiamine hydrochloride, 0.30 g; Pyrodoxine hydrochloride 0.50 g; Folic acid, 0.12 g; Biotin, 0.02 g; Inositol, 23.0 g; Vitamin B_{12} (6 mb/lb), 0.10 lb; Myvamix (Vit. 3), 12.0 g; Klotogen F (10 g synthetic vit. K/lb), 4.0 g; DL Methionine, 0.10 lb.

[a] Present address: Quaker Oats Co., 617 W. Main St., Barrington, Illinois 60010.

Lipids from the lipoproteins and feed samples were extracted with 3:1 chloroform-methanol for two hours on a mechanical shaker at 45°C. The insoluble material was removed by percolating through a cellite plug in a column chromotography tube. Solvent was removed with a rotary evaporator and the lipid sample stored in a screw cap glass vial under nitrogen at 10°F.

Lipoprotein fractionation

The method of Privett et al. (1962) was utilized to separate the triglyceride, lecithin and cephalin fractions of lipids from lipovitellin and lipovitellenin. The fractions were located on the plates by spraying with 0.2% of 2',7'-dichlorofluorescein in ethanol and viewing with ultraviolet light.

Fatty acid composition

The lipid extracts of whole yolk were saponified and free fatty acids were prepared according to Ast (1963). Subsequent methylation of the free fatty acids was accomplished according to the method of Rogozenski (1964).

The methyl esters of the triglyceride, lecithin and cephalin fractions were prepared by scraping their respective "spots" from the thin layer plates into a 100-ml round-bottom flask and refluxing for one hour with 5% sulfuric acid in anhydrous methanol (Evans et al., 1961).

The relative fatty acid distribution was determined with an Aerograph Model A-350-B gas chromatograph using 10 ft-¼-in. stainless steel column packed with 20% diethylene glycol succinate on 60-80 mesh chromosorb P. The column temperature was maintained between 210° and 220°C with a helium flow rate of 125 ml/min.

Lipid content of egg yolk

The total lipid contents of the yolk was determined by a Mojonnier modification of the method for fat by acid hydrolysis (Association of Official Agriculture Chemist, 1960).

Lipid content of egg white

The lipids from the egg white were extracted according to the procedure of Bergquist et al. (1956). The "spread area" of the monomolecular lipid layer was determined on a "measuring tray" according to the method of Colburn et al. (1964).

Sponge cakes

Small sponge cakes, one-egg size (30 g white plus 18 g yolk) were prepared according to the method of Jordan et al. (1960). After removal from the oven, the pans were placed on wire racks, cooled and the cake volume was measured by rape seed displacement. Cakes were then cut and observations made on general appearances.

Emulsification capacity

A modified method of Swift et al. (1961) was utilized to determine the emulsifying capacity of whole egg yolk (Bennett, 1965, personal communication). Corn oil (Mazola) was added drop-wise to a mixture of 0.5 g whole yolk and 15 ml distilled water in a microblender cup of a Waring Blendor. Emulsification capacity was determined as the volume of oil that could be incorporated before disruption of the emulsion occurred.

Table 2—Average fatty acid composition of the total yolk lipid of chickens fed for 20 days on experimental rations.

	Fatty acid (%)								
Ration	12:0[1]	14:0	14:1	16:0	16:1	18:0	18:1	18:2	20:4
LF[2]	—	0.95	—	25.05	4.73	8.48	55.26	4.84	1.04
SO	—	0.38−[3]	—	22.33−	3.02−	7.98	40.64−	22.29+	2.34
HCO	1.04	5.93+	1.66	25.75	5.84	8.37	45.92−	4.95	0.77
CO	—	0.32−	—	23.51	2.79−	6.77	42.70−	21.13+	2.03
OO	—	0.24−	—	22.15−	3.94	4.86	58.13+	9.74	0.92
SFO	—	0.20−	—	23.55	2.38−	8.60	37.15−	27.03+	1.10

[1] Chain length: double bonds.

[2] LF—low fat, SO—soybean oil, HCO—hydrogenated coconut oil, CO—corn oil, OO—olive oil and SFO—safflower oil ration.

[3] (+) indicates a significant increase and a (−) indicates a significant decrease in this value as compared to the low fat ration ($P < 0.05$).

Flavor and mouthfeel of egg yolks

Eggs were gathered during the 7th week on the experimental diets, dipped in mineral oil and stored at 68–75°F in the laboratory. Flavor and mouthfeel of the yolk was evaluated by a taste panel at 0, 1, 2, 4, 8, 12 and 16 weeks. The sample was divided into two parts for duplicate paired comparison with a known fresh standard. The yolk samples were heated to 125°F in a water bath and tasted with glass rod in a dark room equipped with a red light. A "hidden" control was used to determine the reliability of the panel.

RESULTS

Fatty acids composition total egg yolk lipid

The average fatty acid composition of the total yolk lipids after 20 days on the experimental rations is presented in Table 2. Hydrogenated coconut oil in the ration significantly increased the myristic and palmitoleic acid contents and decreased the oleic acid content. The appearance of lauric and myristoleic acid was also noted. Supplementation of the ration with olive oil significantly decreased the myristic, palmitic, palmitoleic and stearic acid content and significantly increased the oleic and linoleic acid content.

Adding safflower oil to the rations resulted in a significant increase in the stearic and linoleic acid content of the total yolk lipids and a significant decrease in palmitic, palmitoleic and oleic acid content.

The addition of corn oil or soybean oil to the ration resulted in similar effects. A significant decrease in the palmitoleic and oleic acid contents and an increase in the linoleic acid content were evident. Soybean oil in the ration significantly decreased the palmitic acid content of egg yolk lipid, whereas the difference was not significant with corn oil.

The major changes in the total yolk lipid occurred in the oleic and linoleic acid content in 9 to 11 days. As linoleic acid content increased with soybean oil, corn oil and safflower oil supplemented rations, the oleic acid content decreased. The linoleic acid content reached a maximum of 22.75% on the corn oil ration and 22.80% on the soybean oil ration. With the safflower oil ration, linoleic acid reached a maximum of 30.05% and oleic acid reached a minimum of 34.75%. The oleic and linoleic acid content increased and decreased, respectively, when the chickens were again placed on the low fat ration, and approached the original level at a slower rate. With hydrogenated coconut oil, the decrease in oleic acid was accompanied by an increase in the shorter chain fatty acids.

The oleic acid content of the total yolk lipids of chickens on the olive oil supplemented ratio increased, leveling off at about 59.95%. No one single fatty acid decreased significantly to compensate for the oleic acid increase. The myristic, palmitic, palmitoleic and stearic acids were all lower in eggs from hens fed the olive oil supplemented ration than eggs from hens fed the low fat ration. The oleic acid content returned to the original level 9 days after the chickens had been returned to the low fat ration.

Lipoproteins of egg yolk

Lipovitellin. The fatty acid composition of the triglyceride, lecithin and cephalin fractions of the lipovitellin is presented in Table 3.

Significant differences in the triglycerides were observed in the levels of myristic, palmitic, stearic and linoleic acids. The myristic acid level was increased greatly by feeding hydrogenated coconut oil, and lauric and myristoleic acids were noted.

The olive oil ration significantly decreased the stearic acid content and increased the linoleic acid contents of the triglyceride fraction. The oleic acid level produced by the olive oil ration was significantly higher than produced by corn oil, soybean oil and safflower oil rations but was not significantly different than that from the low fat ration. Safflower oil

Table 3—Average fatty acid composition of the triglyceride, lechithin and cephalin fractions of lipovitellin of egg yolk from chickens fed for 30 days or more on the experimental rations.

Fraction	Ration	Fatty acid (%) 12:0[1]	14:0	14:1	16:0	16:1	18:0	18:1	18:2	20:4
Triglyceride	LF[2]	—	0.53	—	24.96	5.98	5.94	58.06	4.55	
	SO	—	0.70	—	25.27	4.40	7.30	39.40−[3]	21.18+	
	HCO	1.32	7.19+	2.27	25.54	7.33	5.21	47.63	3.46	
	CO	—	0.67	—	24.08	3.19	6.57	45.64	19.78+	
	OO	—	0.31	—	20.79	3.75	4.05−	62.86	8.23+	
	SFO	—	0.45	—	23.41	3.17	6.73	39.04−	27.20+	
Lecithin	LF	—	0.27	—	35.40	2.75	13.50	43.45	6.40	3.37
	SO	—	0.29	—	31.15	1.30	16.45	25.90−	20.75+	3.85
	HCO	0.46	2.84+	0.83	30.83	3.60	13.41	36.36−	9.63	2.86
	CO	—	0.23	—	31.73	0.85	16.04	23.93−	22.11+	5.22
	OO	—	—	—	31.03	—	13.37	40.57	10.93	4.10
	SFO	—	—	—	31.37	—	17.83+	21.47−	26.80+	3.73
Cephalin	LF	—	0.28	—	12.61	1.44	28.49	30.89	5.80	12.25
	SO	—	—	—	18.49	0.39−	32.31+	19.24−	17.35+	12.25
	HCO	—	0.74+	—	18.54	0.83	28.26	24.50	9.01	16.98
	CO	—	0.20	—	16.99	0.47−	32.85+	17.86−	16.98+	14.93
	OO	—	—	—	16.80	—	30.27+	27.83	8.40	16.73
	SFO	—	—	—	16.53	—	32.53+	15.33−	20.67+	15.00

[1] See Footnote 1, Table 2.
[2] See Footnote 2, Table 2.
[3] See Footnote 3, Table 2.

in the ration significantly decreased the oleic acid and increased the linoleic acid. Corn oil and soybean oil increased the linoleic acid content significantly.

Supplementation with corn oil, olive oil, soybean oil and safflower oil significantly affected the levels of stearic, oleic and linoleic in the lecithin fraction. Safflower oil in the ration significantly increased the stearic and linoleic acid content and decreased the oleic acid content. Likewise, dietary corn oil and soybean oil significantly decreased the oleic acid content and increased the linoleic acid content. Olive oil in the ration also produced a significant increase in the linoleic acid content. Hydrogenated coconut oil significantly increased the myristic and linoleic acid levels and significantly decreased the oleic and arachidonic acid content. Lauric and myristoleic acids were also detected.

Hydrogenated coconut oil in the ration significantly increased the level of myristic and linoleic acids and decreased that of palmitoleic acid in the cephalin fraction. Corn oil, soybean oil and safflower oil rations significantly decreased the palmitoleic and oleic acids and increased the amount of linoleic acids. Olive oil in the ration significantly increased the stearic acid level.

Lipovitellenin. The fatty acid composition of the triglyceride, lecithin and cephalin fractions of lipovitellenin is presented in Table 4.

Significant differences due to dietary treatments were noted in all fatty acids except arachidonic acid in the triglyceride fraction. Olive oil in the ration significantly decreased the levels of myristic, palmitic, palmitoleic and stearic acids and increased the levels of oleic and linoleic acids. Similarly, dietary corn oil and soybean oil significantly decreased palmitic, palmitoleic and oleic acids but not stearic acid and increased the levels of linoleic acid. Safflower oil in the ration produced a significant decrease in the myristic, palmitic, palmitoleic and oleic acid content and increased the content of linoleic acid.

The use of hydrogenated coconut oil ration resulted in a significant increase in the myristic, palmitic and palmitoleic acid levels and a decrease in the oleic acid content of the triglyceride fraction. Lauric and myristoleic acids were also present.

The hydrogenated coconut oil in the ration produced a significant increase in the myristic, stearic and linoleic acid levels of the lecithin fraction and decreased the oleic acid level. Lauric acid and myristoleic acids were also noted. Corn, soybean and safflower oils in the ration significantly decreased the oleic acid content and increased the linoleic and stearic acid levels.

The level of palmitoleic acid in the cephalin fraction was significantly higher with the hydrogenated coconut oil and low fat rations. Corn oil in the ration caused a significant increase in the myristic, palmitoleic and linoleic acids content and a decrease in the oleic acid content. Significant increases in the levels of palmitoleic, stearic and linoleic acids and decrease in the oleic acid content were produced on soybean oil rations.

Safflower oil in the ration significantly increased the linoleic acid content and decreased the oleic acid content of the cephalin fraction. Olive oil in the ration also significantly increased the linoleic acid level but not to as great an extent as corn oil, soybean oil and safflower oil.

Functional properties—emulsification capacity

The average values for emulsification capacity are shown in Table 5. The dietary treatments produced no significant differences in emulsification capacity but there was a significant difference over time during the "changeover" periods of the egg yolk.

Sponge cake volume

The averages of the volumes of sponge cakes are presented in Table 6. Each dietary treatment significantly increased the

Table 4—Average fatty acid composition of the triglyceride, lecithin and cephalin of lipovitellenin of egg yolk from chickens fed for 30 days or more on the experimental rations.

Fraction	Ration	Fatty acid (%) 12:0[1]	14:0	14:1	16:0	16:1	18:0	18:1	18:2	20:4
Triglyceride	LF[2]	—	0.54	—	24.72	6.22	5.22	57.90	5.32	
	SO	—	0.44	—	22.97−[3]	3.81−	5.25	44.36−	22.15+	
	HCO	1.40	7.30+	2.48	26.61+	7.63+	5.06	45.49−	4.34	
	CO	—	0.55	—	23.18−	3.52−	5.47	44.73−	22.69+	
	OO	—	0.32−	—	21.59−	4.20−	3.55−	63.12+	7.18+	
	SFO	—	0.45	—	22.83−	3.35−	6.54	37.91−	28.92+	
Lecithin	LF	—	0.28	—	34.60	2.17	13.08	42.33	7.40	2.15
	SO	—	0.31	—	32.02	1.27	18.80+	23.96−	21.84+	2.78
	HCO	0.34	2.68+	T	31.95	2.43	15.46+	36.70−	9.84+	2.40
	CO	—	0.26	—	32.75	0.69	15.73+	24.91−	22.48+	3.86
	OO	—	—	—	30.17	—	15.03	38.73−	12.30+	3.80
	SFO	—	—	—	29.63	—	15.53+	21.23−	28.00+	5.57
Cephalin	LF	—	—	—	20.11	0.29	25.72	30.34	7.90	15.90
	SO	—	—	—	17.58	1.15+	32.13+	24.47−	16.33+	12.68
	HCO	—	—	—	20.71	0.53+	29.25+	27.98	10.68	13.98
	CO	—	0.85+	—	17.35	1.13+	30.51+	19.42−	17.35+	15.45
	OO	—	—	—	19.03	—	24.97	28.47	10.57	15.97
	SFO	—	—	—	18.57	—	27.33	17.50−	21.47+	15.20

[1] See Footnote 1, Table 2.
[2] See Footnote 2, Table 2.
[3] See Footnote 3, Table 2.

Table 5—Summary of the emulsification capacity of whole egg yolks.

Period	Ration					
	LF[1]	SO	HCO	CO	OO[3]	SFO[3]
0–19th day	50.2[2]	50.3	49.8	50.4	50.2	50.4
37th–58th day	48.9	48.7	48.6	48.9	48.9	48.9
70th–88th day	48.2	48.3	48.1	48.3	48.2	48.1

[1] See Footnote 2, Table 2.
[2] ml of corn oil emulsified by 0.5 g whole yolk in 15 ml H_2O.
[3] Data adjusted for comparative purposes.

Table 6—Summary of the sponge cake volumes made from eggs.

Period	Ration					
	LF[1]	SO	HCO	CO	OO	SFO
0–19th day	289.9[2]	325.6+	309.2	332.9+	314.3+	326.8+
37th–58th day	347.7	361.2+	356.6+	355.8+	325.5	330.0
70th–88th day	301.3	316.6	310.5	312.1	304.5	309.6
Ration Ave.	306.0	329.1	327.0	329.2	313.2	321.7

[1] See Footnote 2, Table 2.
[2] Volume expressed as cc of cake per 80 g batter

volume of the sponge cakes during the first analysis period. The increases in volume produced by the corn, soybean and safflower oils in the rations was significantly greater than those produced by hydrogenated coconut oil and olive oil in the ration, but were not significantly different from each other. The volume of sponge cakes made from eggs from the dietary oil treated groups decreased significantly after the chickens were returned to the low fat ration. The significance was determined by appropriate statistical tests. However, the magnitude of the differences between periods was such as to cause question as to a practical significance in sponge cake volume attributable to dietary treatments.

Generally, the sponge cakes were of good quality with similar texture and crumb color. The experimental rations were semipurified and lacked the pigmented ingredients of normal diets. The color of the yolk progressively decreased to a pale yellow-white during the first two weeks on the experimental feed. The crumb color of the sponge cake also became progressively lighter.

Table 7—Summary of flavor and mouthfeel differences of yolks of eggs stored up to 16 weeks.

Week	LF[1]	SO	HCO	CO	OO	SFO
	Flavor ration					
0	0.50[2]	0.33	0.50	0.50	0.22	0.39
1	0.50	0.56	0.56	0.56	0.40	0.30
2	0.25	0.50	0.38	0.63	0.14	0.21
4	0.65	0.60	0.40	0.50	0.33	0.39
8	0.67	0.39	0.44	0.33	0.31	0.44
12	0.56	0.50	0.61	0.28	0.67	0.75
16	0.60	0.80	0.75	0.70	0.33	0.61
	Mouthfeel ration					
0	0.28	0.16	0.16	0.22	0.38	0.33
1	0.33	0.38	0.44	0.38	0.30	0.25
2	0.13	0.16	0.29	0.44	0.21	0.21
4	0.35	0.40	0.35	0.40	0.61	0.56
8	0.33	0.28	0.50	0.38	0.44	0.63
12	0.72	0.67	0.56	0.61	0.58	0.67
16	0.70	0.80	0.75	0.75	0.39	0.50

[1] See Footnote 2, Table 2.
[2] Percent of "yes there was a difference" with "yes" or "no" being the only choices.

Flavor and mouthfeel

The data in Table 7 summarize the flavor and mouthfeel determination on egg yolk by the taste panel. No significant difference was found in flavor or mouthfeel due to dietary treatment. The flavor of the yolk did not change significantly during storage, whereas the mouthfeel of the yolk from chickens fed the low fat, soybean oil, hydrogenated coconut oil, corn oil and safflower oil became progressively different from the standard egg after storage for varying lengths of time.

Lipid content of whites

The lipid content of white of eggs stored up to 16 weeks at room temperature (68 to 75°F) is presented in Table 8. The differences in lipid content varied little between treatment groups for each storage period. The lipid content increased by 0.0017 to 0.0022% during storage for 16 weeks, but the difference was not significant.

Lipid content of yolks

The lipid content of egg yolks from chickens that had been on the experimental rations was determined. The lipid content of the yolks did not differ significantly regardless of dietary treatment. All were between 32.35 and 32.77%.

DISCUSSION

THE FATTY ACID composition of egg yolk lipids of the hen can be modified by the addition of various fats to their diet. The degree of influence is dependent on the class of yolk lipid, and the fatty acid composition and amount of the fat in the diet. Those fats (soybean oil, corn oil and safflower oil) containing high levels of linoleic acid significantly increased the linoleic acid content of all yolk lipids. The linoleic acid appeared to be selectively deposited at the expense of oleic acid.

Each experimental ration affected the respective lipid fractions of lipovitellin and lipovitellenin in a similar manner. The fatty acid composition of the triglyceride fractions of eggs from hens fed the low fat and hydrogenated coconut oil ration were similar to those reported by Privett et al (1962), and Chen et al. (1965), except for the proportions of oleic and linoleic acid. The fatty acid composition of the triglyceride fractions, which comprise the major portion of the lipids of egg yolk (Evans et al., 1961; Privett et al., 1962) paralleled the fatty acid composition of the total yolk lipid.

Lecithin and cephalin were good carriers of long chain polyunsaturated fatty acids and shorter chain saturated fatty acids regardless of dietary treatment. Lecithin contained a lower proportion of highly polyunsaturated acids and higher proportions of palmitic acid.

No significant differences were found in the fatty acid composition of the total yolk lipids or yolk lipid fractions due to

Table 8—Lipid content of whites from eggs stored up to sixteen weeks (%)

Ration	Weeks						
	0	1	2	4	8	12	16
LF[1]	0.0037	0.0041	0.0043	0.0044	0.0048	0.0051	0.0054
SO	0.0038	0.0040	0.0042	0.0045	0.0049	0.0054	0.0057
HCO	0.0036	0.0039	0.0041	0.0044	0.0049	0.0053	0.0056
CO	0.0037	0.0041	0.0044	0.0045	0.0047	0.0054	0.0058
OO	0.0038	0.0040	0.0043	0.0045	0.0048	0.0052	0.0057
SFO	0.0037	0.0041	0.0043	0.0046	0.0049	0.0055	0.0059

[1] See Footnote 2, Table 2.

the feeding of corn oil as compared to soybean oil. This was expected, since the fatty acid composition of the rations differed only in that the soybean oil ration contained about 7% less linoleic acid, which was compensated for by about 7% linolenic acid.

The safflower oil ration contained 75.9% linoleic acid and resulted in a change of the fatty acid composition of the yolk lipids similar to, but greater in magnitude than that caused by the corn oil and soybean oil rations. The olive oil contained 69.8% oleic acid and the oleic acid content of the total yolk lipid did increase significantly, but not to as great an extent as did the lincleic acid content when corn, soybean or safflower oil were fed.

Lauric acid (47.1%) was the predominant fatty acid in the hydrogenated coconut oil ration. But no increase in the lauric acid content of egg yolk lipids was observed comparable to that of linoleic acid when corn, soybean or safflower oil was fed.

The total lipid content of the egg yolk was not influenced by the supplementation of the ration with the various vegetable oils as has also been observed by other workers (Chung, 1963; Chen et al., 1965).

The lipid content of the whites of eggs, at the beginning of the storage period, corresponded to the yolk content of the whites of fresh eggs reported by Bergquist et al. (1956), Meehan et al. (1962), and Colburn et al. (1964). The fatty acid composition of the yolk lipids apparently had no effect on the transfer of lipid material across the vitellin membrane into the white. The lipid contents of the whites increased at a slower rate than that reported by Meehan et al. (1962) for shell eggs stored at 75°F. The difference in rate is possibly due to the fact that the eggs in the present study were oil-dipped before storage. Smith (1959) states that the quantity of diffused lipid (yolk to white) is proportional to the period of exposure in an environment that promotes moisture loss.

No significant difference was found in the emulsification capacity of whole egg yolk from hens fed rations supplemented with various vegetable oils. This agrees with the findings of Jordan et al. (1962). The differences in emulsification capacity noted over time during certain analysis periods were random, and are believed to be due to uncontrolled factors, such as temperature, that was constant for any one day, but varied between days.

The results of the observations on flavor of the yolks from hens fed the fat-supplemented rations support the finding of earlier workers. The only fat-supplemented rations reported in the literature to affect the flavor of eggs were those that contained linseed oil or cod-liver oil (Cruickshank, 1934; Cruickshank et al., 1939; Albright et al., 1935; and Wheeler et al., 1959).

The significant changes noted in the mouthfeel of the yolks possibly can be attributed to the "natural" thinning noted in storage eggs. Changes in the fatty acid composition of fat also affect its physical characteristics (melting point, firmness), which may also be a contributing factor to the mouthfeel. However, the observations on the mouthfeel of the eggs from treatment groups did not give any indication to this effect.

Jordan et al. (1960 and 1962) found that corn oil added to the rations of hens significantly increased the volume of sponge cakes made from the eggs of those hens. They also reported that the volume of the cakes increased as the length of time on the corn oil ration increased. Procedures followed by Jordan et al. (1960 and 1962) varied from those used in this report in that in their work they blended egg whites from each dietary group and used these whites only in combination with blended yolk from the same dietary treatment in the formulation of sponge cake batter. In the present study, egg white from all treatment groups was blended together. This mixture was used in combination with blended yolk from each dietary treatment. The results of the present study generally agree with the results of Jordan et al. (1960 and 1962). The wide variation in results with all treatment groups over periods has no readily apparent explanation.

No explanation is apparent to account for the increased volume of the sponge cakes made from eggs of the oil treatment groups. The volume increase might be related to the increase in linoleic acid content noted with the corn oil, soybean oil and safflower oil treatment groups. This does not account for the increase in the sponge cake volume with the eggs from the hydrogenated coconut oil and olive oil treatment groups.

Jordan et al. (1960) suggested that perhaps differences in only certain lipids of the egg were involved in differences in functional properties. Examination of the fatty acid composition of the various lipid fractions does not indicate a common factor or tendency that would support their theory.

CONCLUSIONS

1. The major changes in the fatty acid composition occurred in the linoleic, oleic and palmitic acid levels of the total yolk lipids and lipid fractions of the egg yolk lipoproteins of hens fed corn oil, soybean oil, safflower oil and olive oil. The major changes occurred in the shorter chain fatty acids and oleic acids of hens fed hydrogenated coconut oil.

2. The major change in the fatty acid composition (as exemplified with linoleic acid) in the total egg yolk lipids occurred after 9 to 11 days of feeding the hens the oil supplemented ration. Twenty days after the hens had been returned to the low fat ration the total egg yolk lipid had not returned to the pretreatment condition.

3. The triglyceride, lecithin and cephalin fractions of the lipoproteins showed the same relative response to each dietary treatment.

4. The fatty acid composition of the triglyceride fractions of the egg yolk lipoproteins paralleled the fatty acid composition of the total yolk lipids.

5. Lecithin and cephalin fractions of egg yolk lipoprotein are good carriers of long chain polyunsaturated fatty acids and shorter chain saturated fatty acids.

6. No differences due to dietary treatment were observed in emulsification capacity of whole egg yolk.

7. The volumes of sponge cakes made with eggs from hens of the oil-supplemented ration groups were statistically significantly greater than those of the low fat ration group. The practical significance is slight.

8. No difference due to dietary treatment was observed in the flavor or mouthfeel of the yolks of eggs stored up to 16 weeks.

9. No change in flavor of the yolks was observed over the 16 weeks of storage but the mouthfeel of the yolks of eggs from hens fed the low fat, soybean oil, corn oil and safflower oil ration changed over the 16 weeks of storage.

10. No difference in the lipid content of egg white nor in the rate of increase in the lipid content of egg white with storage was observed between the eggs of hens on the dietary treatments.

11. No difference was observed in the yolk lipid content of eggs from hens in the dietary treatments.

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Potentiometric Determination of Fluoride in Beverages by Means of the Ion Selective Solid State Electrode

SUMMARY—A method employing the fluoride ion-selective solid-state membrane electrode has been developed to determine the presence and amount of free fluoride ion in carbonated and noncarbonated beverages. Representative carbonated and non-carbonated beverages containing known amounts of added fluoride were used to relate electrode activity measurements to fluoride concentration in parts per million. Calibration curves were constructed for each system wherein fluoride concentration (ppm), was plotted as a function of potentiometric measurements (millivolts); using the fluoride-ion-activity-electrode as the indicator electrode and a saturated calomel electrode (SCE), as the reference electrode. Beverages studied were selected to represent a range of pH values and complexity of organic and inorganic constituents present in order to demonstrate the ability of the fluoride-ion-electrode to selectively determine free-fluoride ion.

INTRODUCTION

DETERMINATION of fluoride ion concentration in carbonated and noncarbonated beverages has traditionally required distillation as the preliminary separatory procedure. The classical Willard-Winter distillation (1933) as modified into an A.O.A.C. method (1960) has been the basis for most analytical procedures. Spectrophotometric, oscillometric and other procedures are described in review articles written by Fishman et al. (1967). Direct determination of fluoride in water solution has been carried out without preliminary separation of any kind by means of wide line nuclear magnetic resonance by Ferren et al. (1967).

The method described in this paper employs a probe which has been described by Lingane (1967) as being "so specific that fluoride ion activity can be measured as easily as pH is measured with a glass electrode." Our method requires no preliminary separatory step and detects fluoride ion content in complex beverage systems in the range 2 to 0.02 ppm.

Interest in fluoride ion in human nutrition stems from the pioneer efforts of investigators such as Churchill (1931), who pointed out the danger of fluoride in causing mottled teeth enamel. In contrast to this early view, Knutson et al. (1967) stated that systemic administration of fluoride in salt, flour, bread, rice, sugar, milk and other stable foods was most desirable in underdeveloped countries where fluoridated water supplies were not available. The pros and cons of enforced fluoride consumption in water supplies in order to prevent caries has often been an emotional rather than a scientific debate with the vast majority of dental authorities such as Muhlemann (1967) and Gedalia (1967) advocating continued fluoridation of water supplies *plus* the use of auxiliary topical and systemic sources of fluoride. An important consideration in the analysis of fluoride is often whether the fluoride is present as the free available ion. Nuclear magnetic resonance detects *total* fluoride, while the method described in this paper detects only *free available* fluoride ion, and does not measure complexed, precipitated or otherwise unavailable fluoride ion. Beverages were selected to represent commercial soft drinks (wherein the manufacturer has the option to use fluoride-free or fluoride-containing water), and home-made noncarbonated soft drinks produced from packaged powders (wherein the consumer usually uses the readily available tap water).

EXPERIMENTAL

Twelve repetitive measurements of each beverage system containing known amounts

of added sodium fluoride revealed a precision wherein the standard deviation was equal to 0.02%. The 95% confidence limit was found to be ±0.14%, and the relative error of our method compared to the standard distillation method was found to be less than 1%.

Instrumentation

Orion Model 801 Digital pH Meter equipped with the Orion No. 94-09-00 fluoride ion activity electrode was the principal instrument used. Varian PA-7 NMR analyzer was used to provide auxiliary data.

Reagents

Reagents used were sodium fluoride, potassium chloride, potassium acetate (J. T. Baker), Mallinckrodt BuffAR pH 7. Water employed was (1) distilled, (2) well water, (3) municipal fluoridated water. Beverages prepared from pre-packaged powders were made with the various types of water mentioned.

Stock solutions

Stock solutions of sodium fluoride were prepared by diluting 420 mg of sodium fluoride (±0.1 mg) to 1 L in fluoride-free distilled water to yield a solution of 190 ppm fluoride concentration. A series of one to 10 dilutions then yielded a series of sodium fluoride stock solutions at 19, 1.9, 0.19, 0.019 and 0.0019 ppm of free fluoride ion content. These fluoride stock solutions will be referred to as 1 M F^-, 0.1 M F^-, 0.01 M F^-, 0.001 M F^-, 0.0001 M F^- and 0.00001 M F^- or $10^{\circ}MF^-$, $10^{-1}MF$, $10^{-2}MF$, $10^{-3}MF$, $10^{-4}MF$, and $10^{-5}MF$. A stock solution used in our method will be called the "swamping solution" and is made by mixing 100 ml of 1 M potassium acetate; 100 ml of 1 M potassium chloride and 50 ml of Mallinckrodt BuffAR pH 7.

Test solutions

Well water (containing 0.4 ppm of fluoride); municipal water (containing 1.0 ppm of fluoride); commercial carbonated soft drinks; and noncarbonated soft drinks prepared from packaged powders using (a) distilled water, (b) well water and (c) municipal water were used to establish calibration curves after spiking with sodium fluoride. Fluoride content in original system subsequently determined by the procedures described below.

Analytical procedures

Measurements performed with the Orion Model 801 Digital pH meter are very similar in procedure to those required in using any standard pH meter. It is advisable to condition the fluoride ion electrode by allowing it to stand overnight in a solution of distilled water containing the fluoride ion at a concentration within the range of interest. It is also important to maintain consistency of procedure, e.g., (1) stirring of solutions at same rate, (2) maintenance of constant temperature, (3) reading of meter at same time after initial contact with electrodes (approximately 5 min contact insures a stable reading). Details concerning operating procedures and maintenance of the Orion instrument are in literature supplied by the manufacturer. Measurements carried out with the Varian PA-7 NMR analyzer followed procedures described by Ferren et al. (1967).

Sample preparation

Carbonated beverages were de-carbonated by a simple two-step process of (1) gentle heating for several minutes at 60° to 80°C followed by (2) 5 min bubbling with nitrogen gas. Measurements were carried out on systems consisting of one part "swamping solution" (SS), one part test solution (TS) and one part fluoride solution (FS). Calibration curves were obtained by plotting known fluoride content versus millivolts.

RESULTS & DISCUSSION

The use of this method enables one to determine the amount of *available free* fluoride ion in almost any beverage system in the range of 2 ppm and less. Figure 1 illustrates the effect of the diverse ingredients found in a powdered instant soft drink upon fluoride measurements. These ingredients included: fumaric acid, sugar, natural lemon-lime flavor, artificial color, calcium carbonate, dioctyl sodium sulfosuccinate and butylatedhydroxy-anisole. The fact that the distilled water and powdered beverage plots have the same slope indicates that the primary effect is probably due to ionic strength differences in the two systems.

In this preliminary experiment the only variable was the amount of fluoride present while the concentration of other diverse substances remained constant. The Orion electrode measures fluoride ion activity $[F^-]$ and not fluoride ion concentration (F^-). The relationship between activity and concentration of fluoride is stated as being: $[F^-] = \gamma_{F^-}(F^-)$ wherein γ_{F^-} represents the activity coefficient. The activity coefficient varies as a function of *total* ionic strength, μ, as defined by the following formula:

$$\mu = 1/2\sum Z_i^2 C_i$$

where Z_i is the charge of *each* ion present and C_i is the concentration of *each* ion present.

As a matter of practicality, the application of this formula in a literal sense could not be attempted in the present ap-

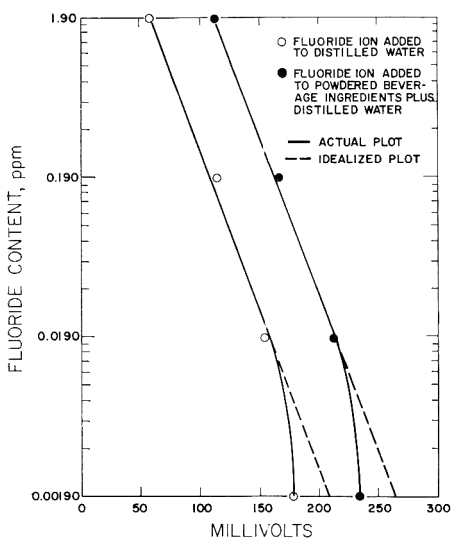

Fig. 1—Fluoride content (ppm) versus potentiometric measurements (mv) made directly in: (1) distilled water and (2) instant soft drink mix ingredients plus distilled water.

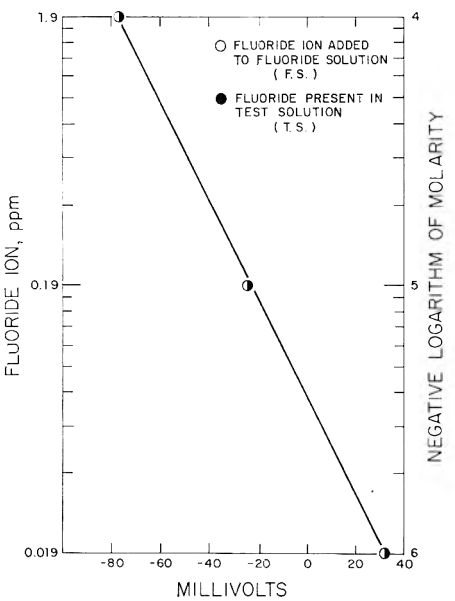

Fig. 2—Fluoride content (ppm) of fluoride stock solution in combination with "swamping solution" and any measured test solution in 1:1:1 ratio by volume.

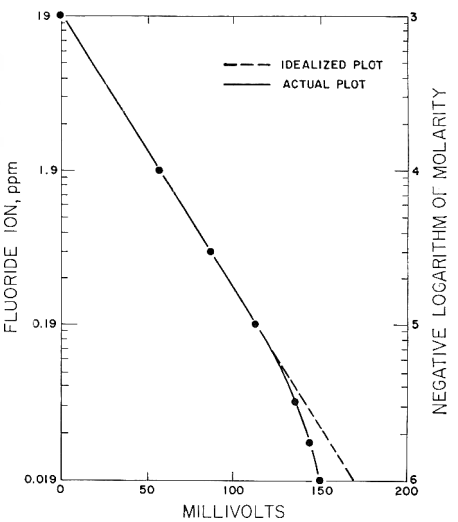

Fig. 3—Fluoride content (ppm) versus potentiometric measurements (mv) in distilled water.

Table 1—Comparison of "free available" and "total" fluoride ion in stannous fluoride and sodium fluoride solutions at the same fluoride ion concentration in distilled water.

Added fluoride ion concentration (ppm)	Calculated NMR signal[a] (Signal height, mm) stannous or sodium fluoride	Potentiometric reading using ion selective electrode[b] (mv) stannous fluoride	sodium fluoride
1.9	2.64×10^{-6}	60.6	62.6
0.19	2.64×10^{-7}	102.5	117.2
0.019	2.64×10^{-8}	129.0	155.0

[a] Identical NMR signal heights obtained for stannous and sodium fluoride solutions at *same* fluoride ion concentration. Values calculated from solutions at fluoride ion concentrations of 0.1*M* and higher concentrations. *Total* fluoride content is measured by the NMR technique.

[b] Different potentiometric readings obtained for stannous and sodium fluoride solutions at *same* added fluoride concentration. Values differed to even greater extent at 0.1*M* and higher concentrations. *Free available* fluoride is measured by this potentiometric technique.

plication. However, the gross application of this relationship was made by means of conductivity measurements. It was found that commercial sodas exhibited measurements in the range of 500 to 1500 mhos, and that the variation between ionic strengths and hence fluoride measurements was reflected in these variances in conductivity. Rather than attempting to adjust each beverage system, the "swamping solution" described under *sample preparation* was discovered to increase the order of magnitude of conductivity to a point at which our procedure yielded an excellent correlation between fluoride content and millivolts for all test solutions examined. At the same time, the use of the incorporated buffer insured the dissociation of all fluoride. As shown in Fig. 2, our method may be employed on a variety of beverages and is not restricted to situations where a known fluoride-free sample is readily available.

Figure 3 illustrates the typical plot of fluoride content versus millivolts obtained by directly adding fluoride ion to distilled water. This type of curve is a useful starting point, but when beverage systems of appreciable ionic strength replace distilled water, a given millivolt reading may no longer be equal to the same fluoride ion concentration. The use of the "swamping solution" removes this restriction and permits one to relate the same millivolt reading to the same fluoride ion concentration for a variety of beverages of diverse ionic strengths.

The method described in this paper is complementary to the wide line NMR method which measures *total* fluoride content. Table I illustrates the utility of both methods in differentiating between *total* and *free available* fluoride content. The precision and accuracy of our method is illustrated by the data shown in Table 2.

Table 2—Comparison of fluoride measurement results.

Sodium fluoride present (ppm)	O = orion electrode method[a] (ppm)	D = distillation method[b] (ppm)
18	22.8	18
	22.3	18
	22.5	19
	22.3	18
	22.8	18
	22.7	
	22.4	
	22.3	
	22.5	
	22.6	
	22.7	
	22.3	
20	20.3	20.2
22	18.1	22.4
24	15.4	23.8
26	13.7	26.0

standard deviation

$$s = \sqrt{\frac{d_1^2 + d_2^2 + d_3^2 \ldots + d_n^2}{N - 1}} = \pm 0.20\%$$

95 percent confidence limit $\pm$ t $s/\sqrt{N} = \pm 0.14\%$

percent relative error $\frac{O - D}{D} \times 100 = 0.8\%$

[a] First example given complete, other values as average of 12 measurements.

[b] First example given complete, other values as average of five determinations.

Our findings may be summarized as follows: (1) Commercial carbonated beverages examined were for all practical purposes devoid of free available fluoride ion; (2) Beverages prepared from packaged ingredients at home using fluoridated water retained the original fluoride content as free available fluoride ion; (3) Milk and orange juice samples examined had fewer than 0.019 ppm as free fluoride ion, but when prepared or spiked with sodium fluoride, the fluoride remained present as free available fluoride ion. The statement of the special joint subcommittee of the Commission on Public Dental Health Services and the Commission on Dental Research (1967) that, "the development and promotion of fluoridated staple foods is particularly urgent and valid for the developing countries," should lead to increased interest as to whether food products and beverages contain free available fluoride ions and the need for methods of fluoride analyses such as the method described in this paper.

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Lethality of Radio-Frequency Energy upon Microorganisms in Liquid, Buffered, and Alcoholic Food Systems

SUMMARY——This work was done to determine if 60 mc/sec R-F energy has a selective killing effect on microorganisms other than that attributable to heat. The organisms studied were Saccharomyces cerevisiae, Escherichia coli, and Bacillus subtilis. An aqueous buffer medium was used to suspend the microorganisms for the irradiation treatments. No killing effect of the R-F energy per se on the organisms was observed at any of the various buffer pH values, nor was there an observable synergistic killing effect of R-F energy and heat on the microorganisms in any of the buffers. However, a synergistic killing effect of ethanol and heat at 48.8°C was demonstrated on S. cerevisiae. Irradiating S. cerevisiae and E. coli in several liquid foods also failed to show a selective killing effect of R-F energy.

INTRODUCTION

THE 1960's have witnessed an intensification of research activities in the area of application of R-F (radio-frequency) energy to the solution of difficult heating problems encountered in the processing of foods. One of the more interesting and important facets of these applications is the possibility of sterilizing or pasteurizing a food product at time-temperature values much lower than those now required using conventional heating techniques. This can be done with R-F energy only if it has a selective killing effect on microorganisms other than that attributable to a heating effect.

This work was done to determine if 60 mc/sec R-F energy has a selective killing effect on microorganisms other than that attributable to heat.

Beckwith et al. (1931) reported significant reductions in the numbers of *Saccharomyces ellipsoideus* and other yeast irradiated with R-F waves. In three cases, sterilization of the yeast suspension was achieved in 15 min. Temperatures of the irradiated suspensions were not allowed to exceed 39°C. Frequency of the radiation was not specified.

Fabian et al. (1933) treated 20-cc broth suspensions of *Escherichia coli* with 7.5, 10, and 15 mc/sec R-F energy in a combination condenser-cooler apparatus which maintained the medium at about 19°C by circulation of cold water in the jacket of the condenser. They found that destruction of the bacteria occurred at the three frequencies with the lethal effect greatest at 10 mc/sec. About 88% destruction of *E. coli* occurred after 8 hr of treatment.

Yen et al. (1934) investigated the effect of R-F waves of 1.5 mc/sec on 10 different strains of bacteria. They exposed the bacteria in saline solution between electrodes in a 15-mm diameter glass tube that was cooled by a coil in the tube through which cold water was circulated. Temperature in the tube never exceeded 20°C. They reported that *E. coli, Bacillus typhosus* (*Salmonella typhosa*), *Bacillus proteus* (*Proteus vulgaris*) and *Bacillus dysenteriae Shiga* (*Shigella dysenteria*) were completely destroyed in 90 min. Four other strains of bacteria exhibited significant decreases in number of viable cells after irradiation for 90 min.

Fleming (1944) irradiated *E. coli* with R-F energy of various frequencies from 11 to 350 mc/sec. The bacteria were suspended in 10 cc of nutrient broth in a tube and placed between the plates of a condenser. A 10-W power input was used and the time of exposure for all treatments was 1 min. Maximum temperature reached during any treatment was 30°C. All frequencies tested had a lethal effect on the bacteria with the greatest effect, about 98% destruction, occurring at approximately 60 mc/sec.

Nyrop (1946) applied R-F energy of 10–100 kc/sec to *E. coli* in broth suspensions. He used electrodes in intimate contact with the broth medium. He observed that 99.6% kill was achieved with a field strength of 205 V/cm in 5-sec and 99.8% kill in 10-sec exposure. There was no marked difference in results when the treatments were made between 12–40 and 40–60°C.

Jacobs et al. (1950) carried out many experiments on *E. coli* and *Staph. aureus* using R-F energy of frequencies from 1.2 to 66 mc/sec. They exposed 6-ml portions of broth cultures of the organisms to R-F energy applied either by two flat electrodes or by a concentric foil electrode. In most of their experiments, no significant killing of the microorganisms was observed. The maximum temperatures reached in the suspensions were about 32°C.

Brown et al. (1954) studied the effect of R-F energy at 50 c/sec, 190 kc/sec, 26 mc/sec on *E. coli*. The bacteria were irradiated in nutrient broth by means of a 6.5-cc capsule electrode assembly. Their initial experiments disclosed many instances of destruction of *E. coli*. They found, however, that a thermal effect was responsible as temperatures in the capsule reached 55°C. They repeated their earlier work and concluded there was no significant killing effect in most treatments unless the final temperature exceeded about 50°C.

Olsen et al. (1966) have reviewed the recent literature on the biological effects of microwave energy. They report that unpublished data of Baker et al. (1965) have shown that microwave energy at 2,450 mc/sec inhibits the germination of macrospores of *Fusarium solani* var. *phaseoli*, probably by affecting a metabolic system in a way distinct from that of heat. Olsen (1965) and Olsen et al. (1966) report that bread mold fungal spores can be eliminated by treatment with microwaves in a conveyor asembly. The temperature of the bread was brought to 65°C in 2 min and cooled to room temperature. When spores of the same fungus were exposed to identical conditions by conventional heating, there was no reduction in spore viability. Thus, they infer that R-F energy has a nonthermal effect on the spores.

Robe (1966) reported on a process for pasteurizing liquids with R-F energy of 27.12 mc/sec which uses a wave guide configuration surrounding a glass or plastic tube through which the liquid flows. Experiments were conducted on beer and wine inoculated with yeast. Sterilization of these products was brought about re-

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peatedly by heating them to 46.2–48.8°C with R-F energy. As 30 min at 60°C is usually required for sterilization using conventional heating, the deduction was made that the R-F energy had a synergistic killing effect with heat on the yeast.

EXPERIMENTAL

Apparatus

Description of radio-frequency apparatus. A schematic diagram of the apparatus is presented in Figure 1. A 500-watt R-F generator having a fixed frequency output of 60 mc/sec was used to supply the R-F energy, which was bound to the medium through a two-electrode capacitor arrangement, with the fluid flowing between the two electrodes as shown in Figure 2. The inner electrode was a solid stainless steel rod, $^1/_4$ in. in diameter by $8^1/_4$ in. long. The outer electrode was a stainless steel sheath, $1^1/_2$ in. inside diameter by 12 in. long.

The inner electrode was connected at one end, through Teflon packing, to the upper plate of a capacitor by a hollow copper tube. The bottom plate of the capacitor was connected to one of the output terminals of the generator. The outer electrode was grounded to the generator chassis and was thereby connected to the other terminal of the generator. Current delivered to the electrodes was varied by increasing or decreasing the spacing between the capacitor plates.

The evaporation chamber was constructed of Pyrex glass and equipped with a mercury-glass thermometer. The remaining components of the R-F evaporation system were constructed of stainless steel and were joined using 1-in. stainless pipe, together with the appropriate elbows and tees. Fittings were the "clamp-type" with a hard rubber, form-fitting gasket between them. The unit was easily disassembled for cleaning after use. An in-line flowmeter was used to measure the flow rate.

Thermocouples and a potentiometer were used to make temperature measurements on the fluid at positions in the area of the electrode. A vacuum tube volt meter, Model 410B, Hewlett-Packard Co., Palo Alto, Cal., was used to measure the voltage difference across the electrodes.

Operation of high-frequency evaporation apparatus. The apparatus was operated in the following manner. A vacuum of about 29.4 in. Hg was drawn on the system by a vacuum pump. The unit was charged by opening the product feed valve and allowing the test fluid to flow into the unit through a submerged glass tube. When the desired operating volume was reached, the recirculating pump was activated and the flow of the liquid adjusted to the desired rate by use of two valves; one on the pump bypass and the other situated in the main line below the flowmeter. The liquid was circulated through the flowmeter into the electrode assembly where the R-F energy was applied. The fluid was then pumped through a nozzle into the flash evaporator. Water vapor was drawn into the condenser, recondensed into water and collected in the receiving flask.

Procedures

Experimental design. Three species of microorganisms were selected for study: *Saccharomyces cerevisiae,* Strain No. 1, obtained from the V.P.I. Department of Bacteriology Culture Collection; *Bacillus subtilis,* Strain No. 1, also from V.P.I.; *Escherichia coli* A.T.C.C., No. 11775, obtained from the American Type Culture Collection, Washington, D. C. They were selected because they are nonpathogenic, easy to culture and each represents a different type of microorganism.

Experiment I. The possible effect of the R-F energy per se on the microorganisms was investigated by irradiating them for 46 min with R-F energy during 2-hour unit operating periods. The experiment was conducted under a partial vacuum at buffer pH values of 7.0, 5.0 and 3.0 for *S. cerevisiae,* and 7.0 and 5.0 for *E. coli* and *B. subtilis.* The evaporation of water from the system at a temperature of about 20°C eliminated the possibility of thermal damage to the microorganisms. Plate counts were made before and after exposure to determine if there was any decrease in the number of microorganisms.

Experiment II. The possible synergistic effect of R-F energy and heat on the microorganisms was investigated at the same buffer pH values as those previously given. The basic procedure was to determine, by water bath studies with a given microorganism, a temperature at which a gradual death phase occurred. The extent of this heat-lethality was determined quantitatively as a function of the pH value of the suspension medium. A corresponding R-F treatment was given to the microorganism at the predetermined temperature. If the R-F energy has a synergistic effect with heat on the microorganism, a greater killing effect than that determined in the water bath studies should be observed.

Experiment III. To check the effect of R-F energy on microorganism in complex media, several studies were made using liquid foods as the suspension media rather than the buffer.

Microbiological methods. A citrate-disodium phosphate buffer in distilled water was selected as the suspension medium for the microorganisms. It can be formulated to any pH value from 2.2 to 8.0. A final concentration of about 0.06 *M* citrate-disodium phosphate was used for all pH values of the buffer.

S. cerevisiae was cultured in malt extract broth (Baltimore Biological Laboratory, Baltimore, Md.) for 48 hr at 26.7°C. The yeast cells were harvested by centrifugation of 200 ml of broth in 250-ml stoppered centrifuge tubes at 900 *g* for 3 min followed by decantation of the supernatant liquid. The cells were resuspended in about 200 ml of 0.06 *M* buffer of the appropriate pH value. *E. coli* was grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) at a temperature of 36.6°C for 24 hr. The cells were harvested by centrifugation and resuspended in 0.06 *M* buffer. The absorbancy of the suspension was adjusted with sterile buffer to a value of 0.1 at a wave length of 590 mμ in a Bausch and Lomb "Spectronic 20" spectrophotometer. Optically matched curvettes 101 mm long and 12 mm I.D. were used.

B. subtilis was grown in tryptic soy broth at 32.2°C for 48 hr. The cells were harvested and resuspended as described for *E. coli,*

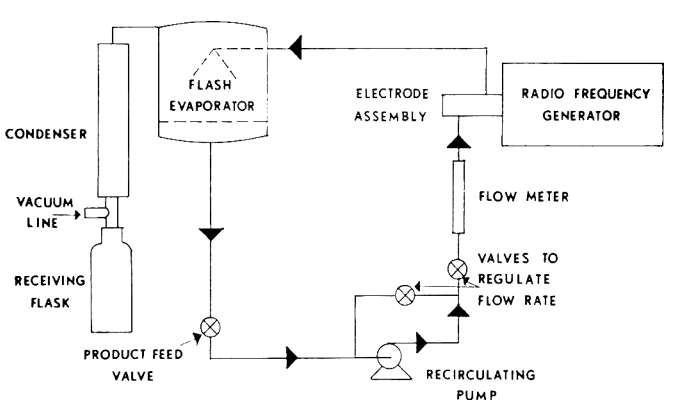

Fig. 1—Schematic diagram of laboratory radio-frequency electronic evaporator.

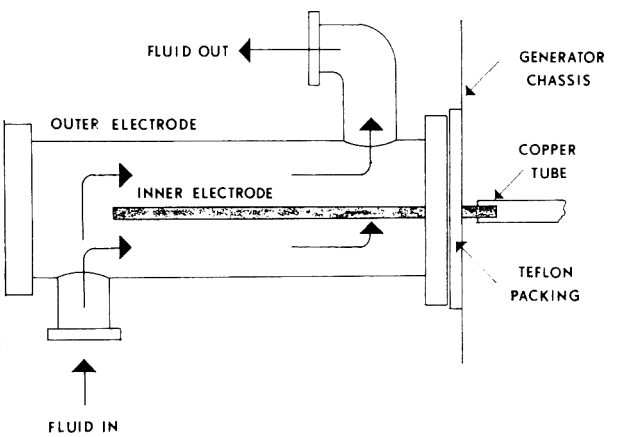

Fig. 2—Diagram of the electrode assembly.

except the absorbancy of the suspension was adjusted to a value of 0.7–0.8. The stock suspensions had the following concentrations of viable microorganisms: *S. cerevisiae*, about 35 million cells/ml; *E. coli*, about 120 million cells/ml; *B. subtilis*, about 25 million cells/ml.

S. cervisiae was plated on Difco potato dextrose agar. Incubation was at 26.7°C for 4–5 days. *E. coli* and *B. subtilis* were plated on Difco Standard plate count agar. Incubation was at 36.6°C for 48 hr.

R-F treatments. Contamination in the apparatus was reduced to an average level of less than two viable microorganisms per ml of circulated buffer by the following procedure. Chlorinated tap water, at the rate of 500 ppm of available chlorine, was circulated through the unit for 10 min. After being drained, the unit was flushed out several times with hot tap water and allowed to drain overnight. Immediately prior to use the next day, the apparatus was flushed out with warm tap water. Finally, water at 210°F was fed into the unit and circulated for 10 min. The unit was drained immediately before use.

Experiment I. To initiate a given treatment, sufficient stock inoculum was introduced into the buffer in the unit to give about a 1:20 final dilution. The buffer was circulated at the maximum flow rate for several min in order to insure thorough dispersion of the cells and then two zero-time control samples were withdrawn into sterile 125-ml bottles. The fluid level in the unit was brought to 3 L and the flow rate was adjusted to about 1.14 L/min which was the slowest rate commensurate with satisfactory functioning of the unit. Therefore, approximately 2.6 min were required for 3 L of fluid to be pumped through the electrode assembly. At this point the R-F generator was turned on and current to the electrodes adjusted to 300 ma. Periodic introduction of sterile distilled water was used to maintain the volume in the unit at a functional level. Liquid level of the system was readjusted to 3 L upon termination of a R-F treatment. Samples of the treated suspension were plated to determine if any destruction of the microorganisms occurred.

Experiment II. This experiment was similar to Experiment I except for the following variations. The temperatures selected at which to study the possible synergistic effect of R-F energy and heat were: 48.8°C for *S. cerevisiae*, 54.5°C for *E. coli*, and 46.1°C for *B. subtilis*. To initiate a treatment, the buffer was heated to the desired temperature in the unit. The inoculum was introduced into the system and allowed to thoroughly mix with the buffer. Zero-time control samples were taken and cooled. Next, the R-F energy was turned on and timing of the exposure was begun.

Temperature of the buffer was held near the desired value, about 0 to −2°C, by regulating manually the vacuum valve to allow distillation of water if the buffer temperature was about to exceed the desired value. The treatments were terminated after various time periods ranging from 16 to 64 min. Samples of about 50 ml volume, to be used for plate counts, were drawn into chilled 125-ml bottles and rapidly cooled to sublethal temperatures by vigorously shaking them in a cold water bath.

Experiment III. Studies were made using tomato juice, milk and orange juice as the suspension medium rather than the buffer.

Freshly made and pasteurized tomato juice was inoculated with *S. cerevisiae* to give a concentration of about 3 million yeast per ml of juice in the evaporator unit. Fresh, whole, pasteurized milk was inoculated with *E. coli* to give a concentration of about 54 million bacteria per ml of milk in the evaporator. Conduct of both determinations was similar to that of Experiment I except that both the tomato juice and the milk were concentrated in the unit to produce 3:1 concentrated products. Whenever the liquid volume in the unit dropped to about 2 L, refrigerated, pasteurized tomato juice or milk was introduced into the unit to restore operating volume. Volume level in the unit was 3 L at the time microbiological samples were withdrawn.

The possible synergistic effect of heat at 48.8°C and R-F energy on *S. cerevisiae* was determined in reconstituted and freshly pasteurized orange juice. Conduct of this determination was similar to that described in Experiment II. The orange juice was inoculated to give a concentration of about 1.7 million yeast per ml of orange juice in the evaporator at the start of the R-F treatment.

Procedure for water bath determinations. Three stoppered Pyrex tubes, 1 in. in diameter and 6 in. in length, were filled with 38 ml of buffer solution, placed in a wire basket assembly, and submerged to ½ in. above the buffer level in the tubes. After the temperature in tubes reached that of the water bath, the determination was initiated by pipetting 2 ml of the stock microorganism suspension into each of three tubes. A fourth tube was likewise inoculated and held at room temperature to serve as a zero-time sample. Samples were withdrawn from the tubes under aseptic conditions after time periods of 4, 8, 16, 24, 32, 40, 48, 56 and 64 min. The tubes were sampled in rotation. A 1-ml sample was withdrawn in the majority of samplings and pipetted directly into either 9 ml or 99 ml of diluent as required, thus insuring nearly instantaneous cooling of the sample to a sublethal temperature.

For samples to be plated undiluted, a volume of about 4 ml was withdrawn using a 10-ml pipette and pipetted directly into a dilution bottle that had been previously chilled to a temperature of −23°C. The bottle was shaken vigorously to speed the cooling of the sample. In these water bath determinations, four replicates were made at each of the various pH values for *S. cerevisiae* and *E. coli*, and two replicates for *B. subtilis*.

Table 1—Effect of 60 mc/sec R-F energy on the survival of S. cerevisiae, E. coli, and B. subtilis at various pH values in 0.06 M citrate-disodium phosphate buffer at 20°C or less under vacuum conditions.

R-F exposure time (min)	Number of viable microorganisms (million/ml)						
	S. cerevisiae			*E. coli*		*B. subtilis*	
	pH 7.0	pH 5.0	pH 3.0	pH 7.0	pH 5.0	pH 7.0	pH 5.0
0	1.775	1.620	1.860	5.450	6.050	1.195	1.310
46	1.765	1.625	1.880	5.300	6.100	1.180	1.315

RESULTS & DISCUSSION

IT WAS considered necessary to include the pH value of the suspension medium as a parameter in this study. The effect of pH in lowering the resistance of microorganisms to heat or to chemical sanitizing agents is a well-known phenomenon. In the literature there has been no mention of investigating the influence of pH. In addition, the media used to suspend the microorganisms during R-F irradiation has, in general, been poorly defined. This situation makes it most difficult to duplicate published experiments and may partially explain the many discrepancies concerning the effects of R-F energy on microorganisms.

For all of the R-F treatments to be discussed, the average temperature rise of the various test fluids per pass through the electrode was about 5°C and the electric field intensity in these fluids ranged from 190 to 205 V.

The results of Experiment I are presented in Table 1. It is apparent that there was no significant decrease in the numbers of microorganisms at any of the buffer pH values tested.

The results of Experiment II are presented in Figures 3, 4 and 5. These graphs show that the decrease in numbers of microorganisms during the combined R-F and heat treatments fall in the range estimated in the water bath studies. Therefore, the unavoidable conclusion is that the only lethal effect on *S. cerevisiae*, *E. coli*, and *B. subtilis* attributable to the R-F treatment is a thermal one and that there was no synergistic effect of heat and R-F energy on these microorganisms.

The unusual configuration of the plots in the case of *B. subtilis* (Fig. 5) can be explained by the presence of spore forms of the organism in the buffer suspensions. The initial slope in the plots results from the death of vegetative cells, while the leveling of the data parallel to the abscissa results from the survival of the spore forms which are known to be very heat resistant.

It was not considered necessary to

replicate all of the treatments in Experiments I and II. However, the treatments at pH 3 for *S. cerevisiae* and at pH 5 for *E. coli* and *B. subtilis* were replicated, and no lethal effects of R-F energy were observed.

The failure to demonstrate a synergistic effect of heat and R-F energy on *S. cerevisiae* is at variance with results previously cited in the liturature (Robe, 1966). As beer contains about 5% ethanol and wine 10–20% ethanol, it was thought that the ethanol content of these products might have contributed with heat in causing the reported lethality. To investigate this possibility, a water bath study was made at 48.8°C at buffer pH values of 7.0, 5.0 and 3.0 in combination with concentrations of ethanol of 2, 6 and 10%. The results presented in graphic form in Figure 6 show that ethanol greatly increased the susceptibility of *S. cerevisiae* to destruction by heat. An additional water bath study, using beer and wine in place of buffer, gave similar results. The beer had an ethanol content of about 5%, a pH of 4.3, and an initial yeast count of 2.330 million cells per ml. The wine was 12% ethanol, had a pH of 3.6 and an initial yeast count of 1.805 million cells per ml. Sterilization was extrapolated to occur at about 4.8 min in the beer and at about 3.9 min in the wine. In the light of these data, there is the possibility that the results presented by Robe (1966) were due to the combined effect of ethanol and heat rather than to that of heat and R-F energy.

The results of Experiment II failed to reveal any lethal effect of R-F energy on *S. cerevisiae* in tomato juice or *E. coli* in milk. There was no reduction in numbers of either microorganism after 3.3 hr of exposure to R-F energy during 9-hr unit operating periods. In addition, the results of the orange juice study failed to reveal a synergistic effect of heat and R-F energy on *S. cerevisiae*. The values for the 16 and 32 min R-F and heat treatments were reasonably close to their corresponding water bath values, i.e., 530,000 viable yeast per ml for the water bath treatment at 16 min vs. 780,000 for the R-F plus heat treatment at 16 min and 5,700 viable yeast per ml for the water bath treatment at 32 min vs. 8,400 viable yeast per

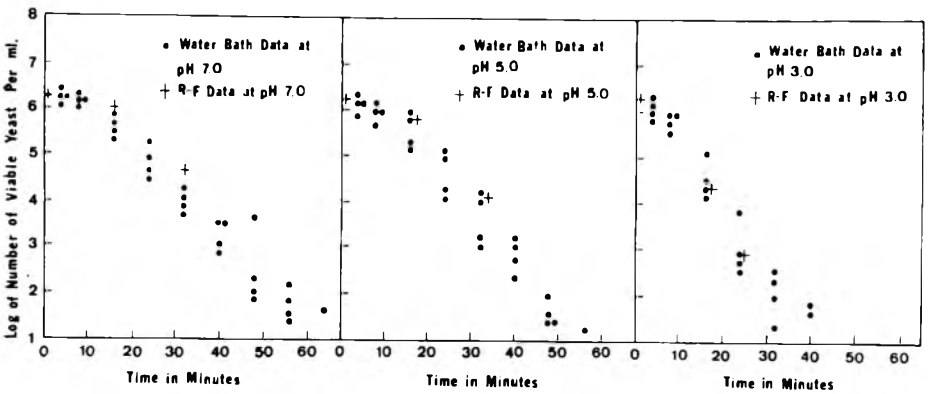

Fig. 3—Synergistic effect of 60 mc/sec R-F energy and heat at 48.8°C on S. cerevisiae *in 0.06 M citrate-disodium phosphate buffer at pH 7.0, 5.0 and 3.0.*

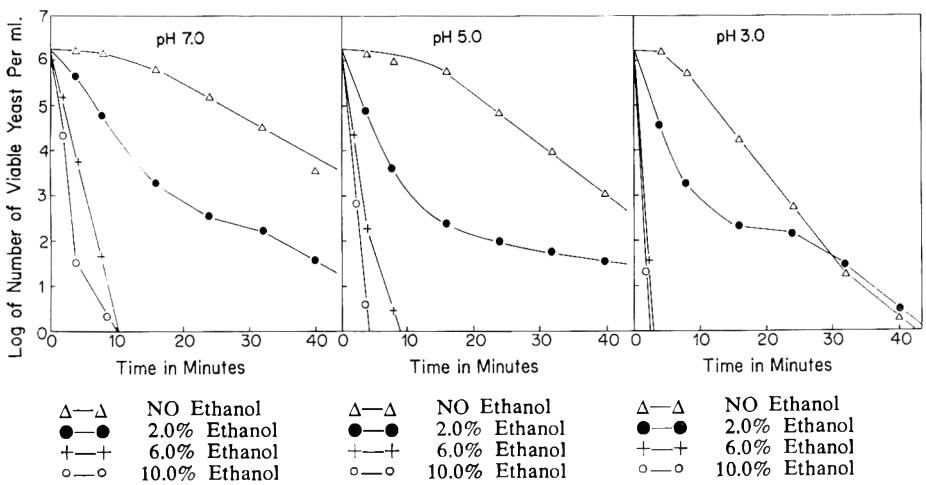

Fig. 4—Synergistic effect of 60 mc/sec R-F energy and heat at 54.5°C on E. coli *in 0.06 M citrate-disodium phosphate buffer at pH 7.0 and 5.0.*

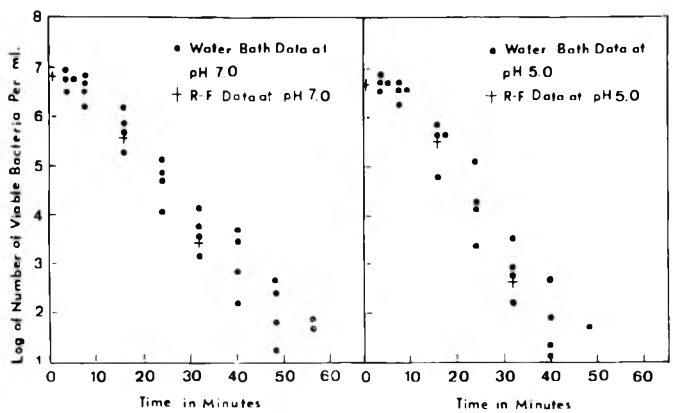

Fig. 5—Synergistic effect of 60 mc/sec R-F energy and heat at 46.1°C on B. subtilis *in 0.6 M citrate-disodium posphate buffer at pH 7.0 and 5.0*

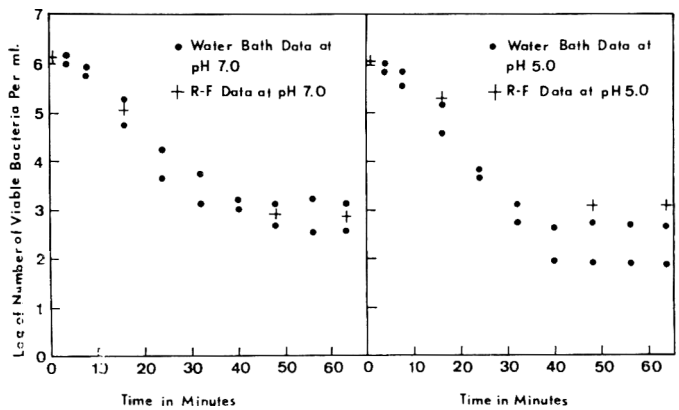

Fig. 6—Effect of ethanol pH 7.0, 5.0, and 3.0 on the death rate of S. cerevisiae *0.06 M citrate-disodium phosphate buffer at 48.8°C.*

ml for the R-F plus heat treatment at 32 min.

The selective destruction of microorganisms through the application of R-F energy could not be demonstrated under the experimental conditions described in this paper. However, the possibility of accomplishing this objective still exists.

Radio-frequency energy is not sensibly "hot"; it is on the contrary, "cold." Heating and other effects occur only through the interaction of the impinging R-F energy and the molecules that constitute the broth medium. With this in mind, there are three principal ways in which R-F energy might cause the destruction of microorganisms.

If heat can be generated faster in the microbial cell than in the suspension medium, the cell might be destroyed thermally at a comparatively low heating rate of the suspension medium. This possibility is dependent upon the chemical composition of the suspension medium and of the microbial cells.

Since most microbial cells bear an electrical charge, usually negative, there exists the possibility of mechanically disrupting the cell by causing it to oscillate rapidly in the high-frequency field. If these oscillations are rapid enough and/or of a large enough displacement, the elastic limits of the cell structure might be exceeded, thus causing the cell to rupture and die. In the work reported in this paper, however, the microbial cells were exposed to a voltage difference across the electrodes of 190–205 V and no lethality was noted.

If R-F energy of a given frequency is selectively absorbed by certain critical organic molecules of the microbial cell, such as an essential protein or DNA molecules, these molecules could be irreversibly denatured and the microorganisms rendered nonviable at low-heating levels of the suspension medium. Whether or not this possibility occurs depends upon what happens on a molecular level. Very little is known concerning the effects of R-F energy on molecules other than water. An investigation into determining a R-F frequency at which DNA molecules resonate could prove especially fruitful. If such a resonance frequency exists, the application of R-F energy of sufficient intensity at this frequency might cause either a direct, physical change in the configuration of the molecule, or the energy could be dissipated mainly as heat. Either or both occurrences might be of large enough magnitude to cause the irreversible denaturation of the molecule.

The authors are unable to explain away the killing effects of R-F energy per se reported by other workers, notably Beckwith et al. (1931), Fabian et al. (1933), Yen et al. (1934), Fleming (1944), and Nyrop (1946). Aside from the differences in frequencies employed, there is the possibility that localized overheating or some experimental artifact might be responsible for the observed lethalities. These workers treated small quantities of fluid, on the order of 5–20 ml, and did not continuously monitor temperature readings.

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Reaction at Limited Water Concentration 1. Sucrose Hydrolysis

SUMMARY—To enable development of a model describing reaction kinetics in dehydrated foods, we studied sucrose hydrolysis at limited water concentration. Saturated sucrose solutions containing various acids and inert solid materials gave identical rate constants and energy of activation as predicted from dilute solutions. Reaction rates in freeze-dried systems humidified to low moisture contents indicated that any equation describing the rate of hydrolysis must include a term for the velocity of dissolution of solid sucrose into the surface water. The rate of hydrolysis was a pseudo first-order reaction obeying the same kinetics as in dilute solution; the rate of dissolution became rate-limiting when the initial supply of dissolved sucrose was exhausted.

INTRODUCTION

THE RATE of various deteriorative reactions which occur in dehydrated foods is usually a function of the amount and state of water in the food. There have been extensive investigations of the mechanism of enzymatic reactions (Acker, 1963), lipid oxidation (Maloney et al., 1966; Labuza et al., 1966), and nonenzymatic browning (Lea, 1958; Tannenbaum, 1966) in dehydrated systems. These investigators stressed the importance of the interaction between water and the various reactants. The similarities between dehydration and freezing (Fennema, 1966) as methods of limiting water concentration are significant.

With nonenzymatic browning of sugar-amino systems, Hodge (1953) has shown that a carbonyl group on the sugar is nec-

essary for the reaction to occur. However, the recent work of Karel et al. (1967) shows that in a dehydrated system containing the nonreducing sugar sucrose and citric acid at low water activity, an appreciable browning reaction can occur. Their data indicate that the browning reaction was preceded by hydrolysis of sucrose. This hydrolysis formed fructose and glucose, which are reducing sugars. In the present study, the mechanism of the sucrose hydrolysis reaction was studied in systems at limited water concentration. The results could be used both to explain the browning of nonreducing-sugar systems as well as to increase the knowledge about the state of water at low activity.

THEORETICAL

General mechanism

The rate of sucrose hydrolysis is a function of the concentrations of reactants, temperature and acid-catalyst concentration. The stoichiometric reaction is:

$$\text{Sucrose} + H_2O \xrightarrow{H+} \text{Fructose} + \text{Glucose}$$

(Glasstone, 1946). Under these conditions the rate of disappearance of sucrose or appearance of reducing sugar is:

$$\frac{-d(S_t)}{dt} = +\frac{dX}{dt} = k(S_0 - X)(W_0 - X) \quad (1)$$

in which

S_0 = initial sucrose concentration
S_t = sucrose concentration at any time
$S_0 - X$ = sucrose concentration at time t
W_0 = initial water concentration
X = reducing sugar concentration (equals glucose + fructose)
t = time, and
k = rate constant.

Integration of Eq (1) gives this second-order equation:

$$\frac{1}{S_0 - W_0} \ln \frac{W_0[(S_0 - X)]}{S_0[(W_0 - X)]} = kt \quad (2)$$

However, if water is present in great excess, the fraction of water used in the reaction is negligible. Thus the water value can be included in the constant k, and Eq (1) can be integrated to give the pseudo first-order reaction-rate equation:

$$\ln \frac{S_0}{S_0 - X} = kt \quad (3)$$

A semi-log plot of $S_0/S_0 - X$ vs. time would therefore be a straight line whose slope is the observed first-order rate constant k. When the water concentration is in excess, the data could then be plotted both as a first- or second-order reaction. When water becomes limiting, as might be expected at high sucrose concentration and low water activity, only the second-order rate reaction should apply to the data.

The rate constant k can be divided into its component parts of temperature dependence, water concentration and catalyst concentration. After reviewing the literature on sucrose hydrolysis in dilute solution, Vukov (1965) developed this empirical equation for the rate constant:

$$\log_{10}(k) = 16.91 + \log_{10}(W_0) - \frac{5670}{T} - \text{pH} \quad (4)$$

in which

k = measured rate constant
W_0 = initial water concentration
T = absolute temperature.

Thus, the log of the rate constant is proportional to the inverse of the absolute temperature and directly proportional to the pH.

Hydrolysis in a "dry" system

Water in the hydrolysis reaction serves both as a substrate as well as a medium for reaction. At low water activity (low relative humidity) and on a soluble solid substrate, the state of water should control the fraction of the total quantity of substrate that can participate in the hydrolysis reaction. The moisture isotherm of the material would give a picture of the state of water by dividing it into tightly bound water below the monolayer, loosely bound multilayer water, and free water of capillary condensation (Adamson, 1960). The extent to which the pseudo first-order kinetic model described above is consistent with the experimental data should give information on the availability of water at low activity, since when water becomes a limiting reactant the kinetics will no longer be first order.

If excess solid sucrose is present in a saturated sucrose solution undergoing hydrolysis, a diffusion gradient will exist between the solid surface and the lower concentration in the aqueous media surrounding it, once hydrolysis occurs. Thus, solid sucrose should dissolve to maintain the concentration at the saturation value. However, as hydrolysis occurs, the concentration of invert sugar increases and thus depresses the solubility of sucrose and establishes a lower saturation value. The data of Jackson et al. (1924) are interpolated at 37°C to show this effect and are presented in Fig. 1.

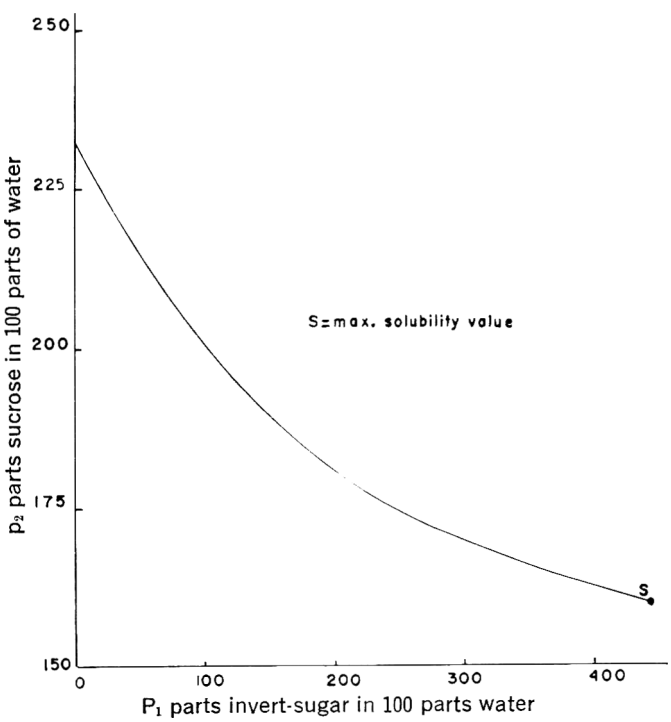


Fig. 1—Effect of invert-sugar concentration on solubility of sucrose at 37°C.

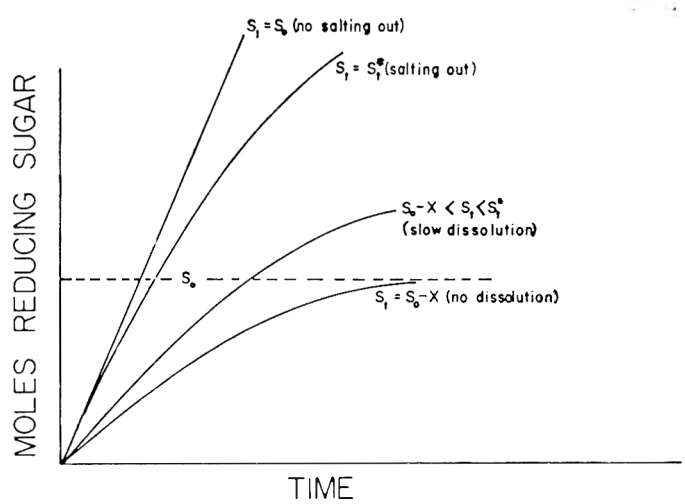
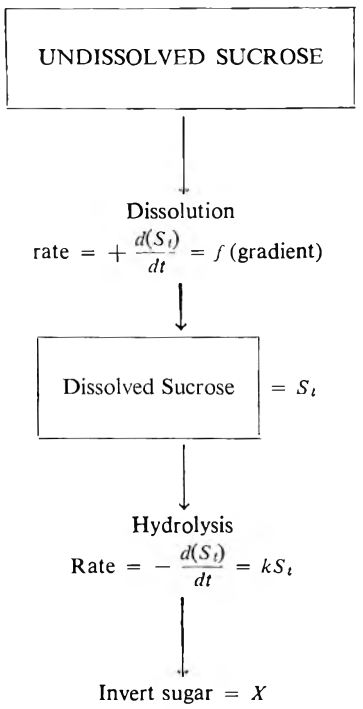


Fig. 2—Theoretical: Reducing-sugar formation in systems at limited water concentration.

Under these conditions, the overall kinetic pattern for sucrose hydrolysis in the presence of solid sucrose can be represented as follows:



There are 3 possible cases for this model:

(a) The rate of dissolution is greater or equal to the rate of hydrolysis. Thus, the aqueous phase always remains at the saturation value S_t* which decreases as X increases:

$$S_t = S_t^* \qquad \text{Case a}$$

(b) The rate of dissolution is negligible compared to the rate of hydrolysis:

$$S_t = S_0 - X \qquad \text{Case b}$$

(c) A condition between the 2 extremes in which the dissolution rate is small, but not negligible:

$$S_0 - X < S_t < S_t^* \qquad \text{Case c}$$

These 3 conditions are represented graphically in Fig. 2. In all cases the effect of water depletion is neglected.

MATERIALS & METHODS

Model systems

Four types of model systems were prepared as follows:

System I. The exact amount of sucrose necessary to make a saturated solution (Bates, 1942) is added to water at room temperature with continuous stirring on a stirrer hot plate. After covering the beaker with Parafilm, we heated the solution to not more than 5°C above the desired temperature to complete dissolution. The required amount of acid was then added and dissolved. Under these conditions water loss and hydrolysis were negligible.

System II. A known amount of system I was mixed with microcrystalline cellulose (Avicel, American Viscose Co., Marcus Hook, Pa.) in the ratio of 50 g microcrystalline cellulose to 100 g water in a Sorvall Omni-Mixer mixing cup (full speed for 5 min). The mixing was done in an ice bath to prevent heating.

System III. To a known amount of system II, sucrose crystals were added and mixed in by hand until a homogeneous mixture was obtained. Enough excess crystals were added so that an excess was always present during the hydrolysis reaction.

System IV. Samples of about 35 g of system II were transferred into 125-ml flasks, frozen in liquid nitrogen, and then freeze-dried at 20 μHg for 96 hr at 80°F. After freeze-drying, the samples were equilibrated to 75% relative humidity over saturated sodium chloride solution for 5 hr.

Hydrolysis

After preparation the samples were placed in covered containers in a water bath maintained to within ±0.01°C. The extent of hydrolysis was measured in 2 ways:

Reducing-sugar test. A modified Somogyi procedure (Bates, 1942) was used, after dissolving a known weight of the test sample. Care was taken to prepare dilutions in the proper range to insure accuracy of measurement. The standard curve was prepared by using equimolar amounts of glucose and fructose since their individual reducing properties differ slightly.

Polarimetric method (Bates, 1942). We used the standard polarimetric technique in which the measure of the change in optical rotation of a solution can be related to the concentration of the various optically active components in the system. The test system was dissolved in water to a suitable dilution and filtered, when microcrystalline cellulose was present, to give a clear solution. A Hilger and Watts polarimeter (model C412), giving an accuracy of ±0.01°, was used with a 20-cm jacketed tube.

When the microcrystalline cellulose was present in the system, an additional rotation of 0.7—1.50° was obtained over that of pure sucrose (21.90°). In all cases, therefore, the polarimetric results were corrected for this additional rotation. No other additives contributed to specific rotation.

The extent of hydrolysis (H%) is expressed in the Results section as the moles of reducing sugar (1 mole reducing sugar = 1 mole glucose + 1 mole fructose) divided by the initial sucrose concentration.

Where possible, pH was measured with a Corning (model 12) pH meter by use of a microelectrode.

Table 1—Saturated-sucrose solution runs[1]

Run	Temperature (°C)	Sucrose	Acid used	pH average	$k \times 10^3$
1	25	211	5 g citric	1.70	2.41
2	25	211	10 g citric	1.52	3.54
4, 5, 6	37	232	5 g citric	1.80	13.84
7, 8, 9	37	232	10 g citric	1.60	20.20
10	38.5	232[2]	5 g citric	1.83	17.30
11	38.5	232[2]	10 g citric	1.63	25.32
12	37	232	5 g adipic	2.63	2.1
13	37	232	0.01*N* HCl	1.68	21.4
14	37	232	0.001*N* HCl	2.76	1.67
15[3]	37	232	5 g citric	1.92	10.1
16[3]	37	232	10 g citric	1.70	16.3

[1] 100 g water/system.
[2] Slightly below saturation value of 235 g for 38.5°C.
[3] Contains in addition 100 g microcrystalline cellulose (system II).

Table 2—Activation energy for sucrose hydrolysis

Dilute solution	Reference
E_a kcal/mole	
26.0	Sizer (1937)
25.7	Heidt (1938)
25.8	Lamble et al. (1915)
	Moelwyn et al. (1930)
28.2	Bodamer et al. (1951)
Saturated solution	
26.3 (5 g citric acid)	Present study
26.6 (10 g citric acid)	Present study

RESULTS & DISCUSSION

Saturated sucrose solutions

Saturated sucrose solutions of system I were prepared according to the composition shown in Table 1. The extent of hydrolysis and the variation of pH were measured as a function of time. An example of the data is shown in Fig. 3 for extent of hydrolysis. The pH was found to decrease slightly during the experiment, and an average pH was determined from the time during which approximately 40–50% of the hydrolysis occurred (Table 1).

The system I solutions, although containing only about 8 moles water/mole sucrose, clearly exhibited a pseudo first-order reaction rate as shown in Fig. 4 for the runs at 37°C. Even with close to 90% hydrolysis, the 10% decrease in water concentration did not affect the rate equation. The close agreement of the polarimetric and reducing-sugar tests (Fig. 3) indicated that browning had not yet occurred. The calculated rate constants for system I are tabulated in Table 1. The calculated activation energy was 26.3–26.6 kcal/mole. Values of activation energy in dilute solution vary in the literature but are very close to the present results for saturated solutions. The results are tabulated in Table 2 for comparison.

The variation of the rate constant in dilute solution with pH has been shown previously in equation (4). A semi-log plot of k vs. pH should be a straight line with a slope equal to one. The average estimated pH values vs. the measured rate constant at 37°C plotted in Fig. 5 show that the saturated solution also follows this equation. The points for hydrochloric acid fell slightly above the line; this was probably due to the slight catalytic effect of the chloride ion (Hammet et al., 1933). From equation (4) for hydrochloric acid at pH 2 in a saturated solution at 37°C,

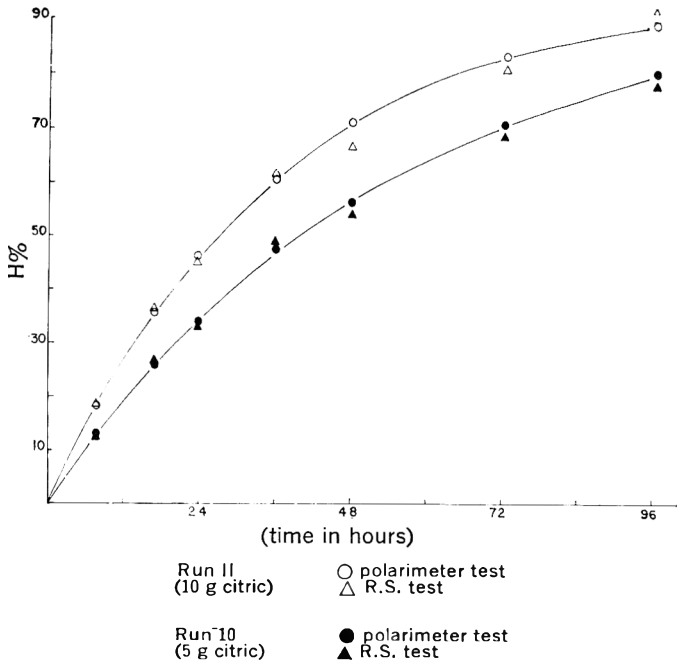


Fig. 3—Extent of sucrose hydrolysis in a saturated solution as a function of time at 38.5°C

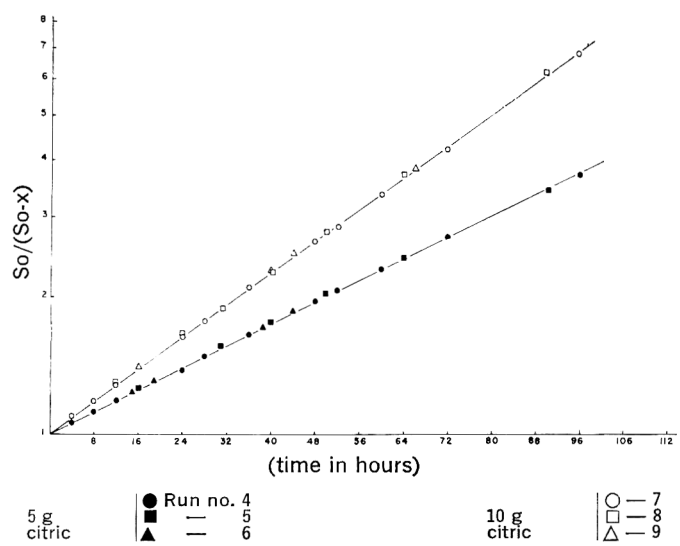


Fig. 4—First-order rate plot from sucrose hydrolysis in a saturated solution at 37°C.

the calculated value of $k = 10.5 \times 10^{-3}$ hr^{-1} compares favorably with the data of Fig. 5.

The composition for system II containing added microcrystalline cellulose (runs 15, 16) are presented in Table 1. The hydrolysis data at 37°C followed observed first-order kinetics (Fig. 6), and no surface catalytic effect of the cellulose was found. The major effect of the cellulose is to increase the pH slightly by a buffering action (Fig. 5). In the presence of cellulose the pH was constant throughout the hydrolysis reaction due to this buffering action.

Saturated sucrose solutions with excess solid sucrose

Excess solid sucrose can affect the rate of hydrolysis as suggested in the Theoretical section. Two runs were made at 37°C with the compositions shown in Table 3. The extent of hydrolysis, pH and the concentration of dissolved sucrose in the aqueous phase was measured with time. This latter test was made by centrifugation of the sample at 27,000 *g* and measurement of the total reducing sugars in the supernatant before and after complete hydrolysis with hydrochloric acid. From this data, the actual concentration of sucrose in the aqueous phase can be compared to:

(a) S_t*—the saturated solution concentration of sucrose assuming a rapid dissolution rate of solid sucrose (corrected for reducing-sugar salting-out effect).

(b) $S_0 - X$—the concentration of su-

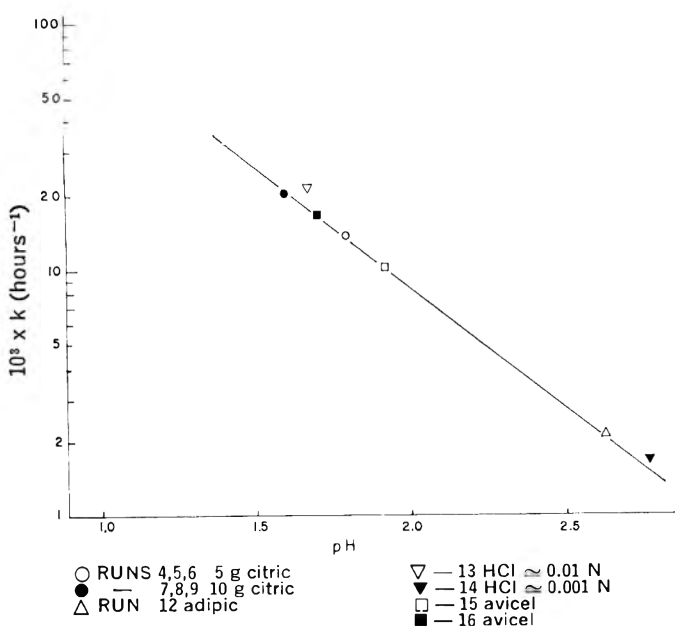


Fig. 5—Dependence of first-order rate constant on pH for sucrose hydrolysis in a saturated solution at 37°C.

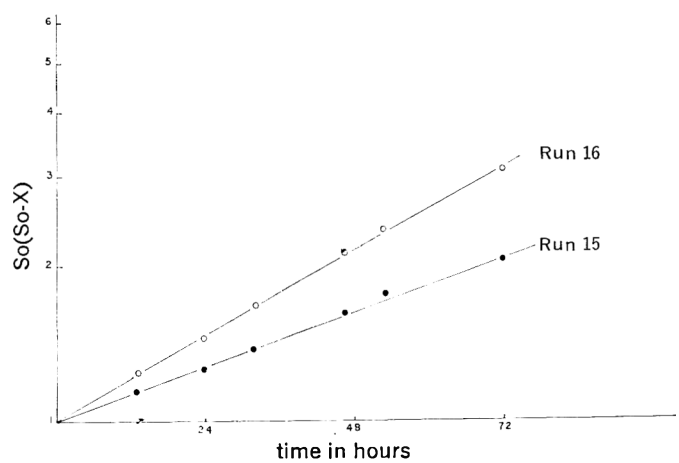


Fig. 6—Effect of the presence of an inert solid on sucrose hydrolysis in a saturated solution at 37°C. First-order rate plot.

Table 3—Composition: saturated-sucrose solution runs with excess sucrose (temperature = 37°C)

	Run 18	Run 19
Total sucrose (g)	392	537
Citric acid (g)	10	10
Microcrystalline cellulose (g)	50	50
Water (g)	100	100
Dissolved sucrose		
S_0 (moles/100 g solids)	0.150	0.114
W_0 (moles/100 g solids)	1.230	0.933
pH (average)	1.75	1.73
$k \times 10^3$ (hr^{-1})	14.9	16.2

Table 4—Hydrolysis in a saturated-sucrose solution in the presence of undissolved sucrose (run 18)

Time	(moles/100 g solids)			
(hr)	X[1]	S_t[2]	$S_0 - X$[3]	S_t*[4]
24	4.8	9.6	10.2	13.5
48	7.9	7.0	7.1	12.8
72	9.9	5.6	5.2	12.0
96	11.4	3.8	3.6	11.5

[1] Measured amount of reducing sugars.
[2] Measured sucrose concentration.
[3] Calculated sucrose concentration. No dissolution of excess sucrose.
[4] Calculated sucrose concentration. Rapid dissolution of excess sucrose.

crose in solution assuming no dissolution of solid sucrose.

As an example, these values are shown in Table 4 for run 18. The data clearly indicate no measurable dissolution of excess sucrose. Similar data were obtained for run 19. Under these conditions, the data obey the first-order rate equation (3) and are consistent with a model of case b. The rate constants shown in Table 3 fall on the line of Fig. 5 for the average measured pH value, and thus indicate conclusively that the excess sucrose had no surface catalytic effect.

Hydrolysis in humidified freeze-dried model systems

The major problem in attempting a kinetic analysis of sucrose systems undergoing hydrolysis at a very low moisture content is that it is not possible to measure directly both the portion of the water available for dissolution of sugar and the pH of this solution. Two runs were made at 37°C with system IV models in which 10 g of citric acid was the catalyst, and hydrolysis was measured as before.

The measured initial moisture content for the samples prepared for run 20 was 3.1 g H_2O/100 g solids. The extent of hydrolysis for run 20 is shown as a function of time in Fig. 7. The data show that even at this moisture content, hydrolysis can occur. In order to analyze the kinetics of this reaction, it was initially presumed that all the water is available for solution even though this water may be held tightly. This amount of water then gives the value of S_0 as shown in Fig. 7. Assuming the same conditions as in analysis of system III, and using the calculated hydrolysis rate constant for the first 10 hr (under this condition the amount of hydrolysis is very small so the concentration gradient for dissolution is negligible), we show the theoretical curves for no dissolution (Case b) and maximum dissolution (Case a) of solid sucrose in Fig. 7. In run 21, the sucrose crystallized as a monohydrate with a water content of 4.2 g/100 g solids at <0.1% RH. If the equilibrium water content of this system at 75% RH (6.3 g H_2O/100 g solids) is corrected for the mole of water per mole of sucrose unavailable for reaction, then the initial sucrose concentration is $(S_0)_2 = 1.65 \times 10^{-2}$ moles/100 g solids. The uncorrected value is $(S_0)_1 = 4.3 \times 10^{-2}$ moles/100 g solids. The extent of hydrolysis with time and the kinetic parameters discussed for run 20 are shown in Fig. 8. The data for these 2 runs suggest the following:

1. Sucrose hydrolysis can occur in systems in which the water activity is low. Water of crystallization, however, is not available for this reaction.
2. As dissolved sucrose is hydrolyzed, dissolution of sucrose occurs into the aqueous phase. The reason for the measurable dissolution rate under these conditions as compared to the system III saturated-solution models in which no dissolution was found is probably due to the solid surface area to liquid volume ratio. At low moisture contents, this ratio is very large, and since the amount of dissolution should be proportional to solid surface area, a proportionally larger quantity of sucrose dissolves. In our tests for both runs the initial amount of sucrose was hydrolyzed in 80 hr.
3. The initial rate constants predict a pH of 1.58–1.64, which is not unreasonable to assume for the aqueous phase on the solid surface. Our results (Fig. 5) for run 20 were $k = 19.2 \times 10^{-3}$ hr^{-1} and run 21, $k = 21.8 \times 10^{-3}$ hr^{-1}.

CONCLUSIONS

This study indicates that sucrose hydrolysis in saturated solutions obeys the same kinetics as that in dilute solutions. Catalysis by inert solids is negligible and dissolution of excess solid sucrose occurs

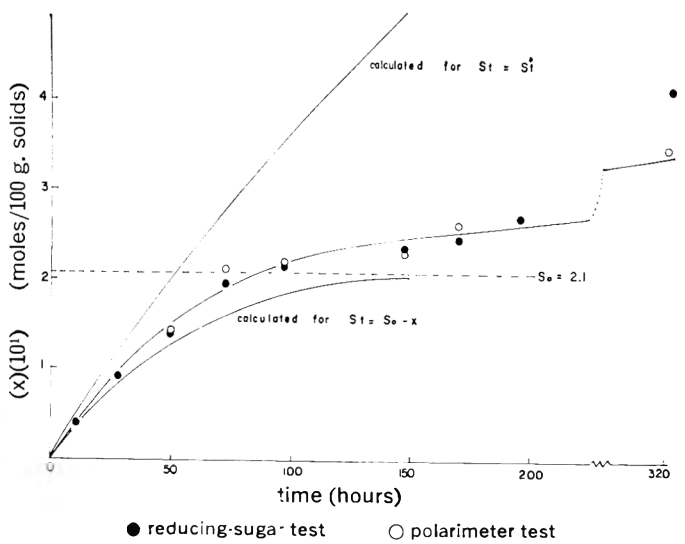


Fig. 7—Extent of sucrose hydrolysis in a freeze-dried system at limited water concentration (3.1 g H_2O/100 g solids) at 37°C.

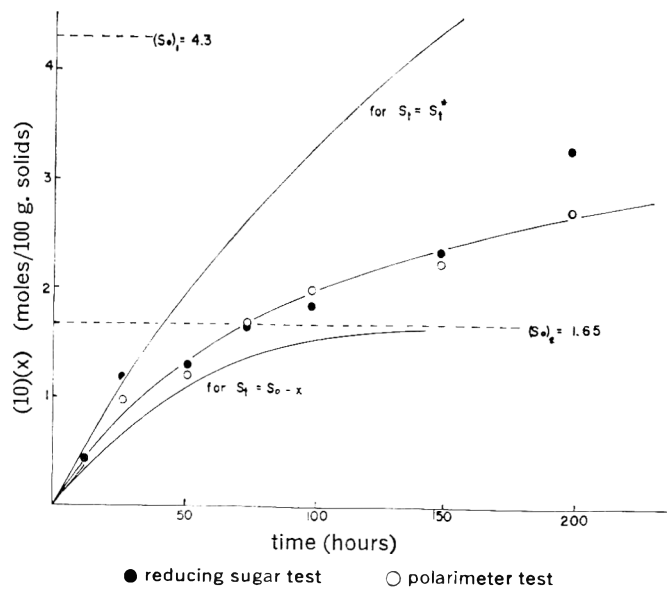


Fig. 8—Extent of sucrose hydrolysis in a freeze-dried system at limited water concentration (6.3 g H_2O/100 g solids) at 37°C.

so slowly that there is no effect on the normal kinetics. At very low moisture contents, the data indicate that the same kinetics of hydrolysis occur in the small amount of adsorbed water as in saturated solutions. This observation shows that the adsorbed water is readily available for reaction. It was also shown that dissolution of solid sucrose occurs at a measurable rate when the solid-sucrose surface area to liquid volume ratio is large.

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Free Amino Acids in Bovine Muscles and Their Relationship to Tenderness

SUMMARY—Free amino acid (FAA) analyses were conducted on 87 l. dorsi and 58 b. femoris samples from cattle varying in sex, breeding and age to study the relationships between these muscle constituents and Warner-Bratzler Shear values. The b. femoris contained greater amounts of FAA than the l. dorsi and was significantly tougher. Muscles from a line of cattle that tended to be more tender possessed greater amounts of FAA. With few exceptions the l. dorsi of steers contained greater amounts of FAA than the same muscle from bulls. Differences in bull muscles due to age were not significant for any FAA. Steer muscle with low shear values tended to possess greater amounts of FAA than steers with high shear values. Although not significantly correlated individual and total FAA increased slightly with increasing tenderness within beef muscles.

INTRODUCTION

THE TOTAL amino acid composition of muscle protein is quite constant regardless of the species or the muscle from which it is obtained (Lyman et al., 1949; Schweigert et al., 1945; Blum et al., 1966). Nevertheless, free amino acid composition of muscle has been reported to vary with aging (Niewiarowicz, 1958; Colombo et al., 1958), cooking (Ginger et al., 1954; Zoltowska, 1967), and frozen storage time and temperature (Golovkin et al., 1966). Ma et al. (1961) and Usborne (1967) have shown that greater amounts of certain free amino acids are related to more tender beef and pork, respectively. Miller et al. (1965), however, found no relation between tenderness and the general pattern of free amino acid concentration or between tenderness and the concentration of any single free amino acid in chicken muscle. McClain et al. (1968) reported that correlation coefficients between 12 of the free amino acids in aged ham muscle and aged flavor were highly significant. They referred to work by several investigators who suggested that certain amino acids are important precursors of the flavor components released when foods are heated.

The purpose of this experiment was to study variation in free amino acids due to muscle, sex, age and line of beef cattle and to relate free amino acid composition to tenderness of beef after a constant aging period. The information obtained may also be of value in interpreting flavor

differences in beef muscle.

EXPERIMENTAL

FREE amino acids were determined in the longissimus dorsi (l. dorsi) and biceps femoris (b. femoris) muscles from 34 good and choice bull carcasses approximately 14 months of age, and from 24 good and choice bull carcasses approximately 20 months of age. All bulls had received similar rations. The carcasses were from a line of inbred Herefords that had been selected for rate of gain for 13 years; a line of Angus; and a second line of Herefords. Number of carcasses were approximately evenly distributed with respect to age and lines. Free amino acids were also determined in the l. dorsi muscle of 29 choice grade Hereford steer carcasses approximately 20 months of age. These were selected as the 14 most tender and the 15 least tender from a group of 109 steer carcasses.

All carcasses were aged for 7 days after slaughter at approximately 40°F. The epimysium was removed from l. dorsi and b. femoris steaks cut from the 12th rib section and the dorsal end of the bottom round, respectively. Samples from these steaks were then ground through a $^1/_8$-in. diameter plate and frozen for 4 to 6 months. Adjacent steaks were frozen for 4 to 6 months, thawed at room temperature and roasted in a 350°F oven to an internal temperature of 160°F. The Warner-Bratzler Shear was used as an objective measure of tenderness of the cooked meat. Three 1-in. cores cut parallel with the muscle fibers were taken from the lateral, central and medial positions of the l. dorsi and b. femoris muscles. Each core was sheared three times.

The ground muscle samples were removed from the freezer and allowed to thaw at room temperature approximately 1 hour. Deproteinization of the meat samples was carried out by a modified method of Stein et al. (1954). Six to 7 g of muscle were then homogenized with 100 ml of 1% picric acid. The samples were agitated, centrifuged and the supernatant poured off and filtered. The remaining precipitate was then washed twice with 10 ml of 1% picric acid, centrifuged and filtered. The washings, combined with the original filtrate, were passed through a column containing a Dowex 2-x8 (chloride form) resin bed. Prior to use, the resin was activated with 1 *N* HCl and then washed with distilled water until the filtrate was neutral. The resin was packed 3 cm long in a 2 × 25 cm chromatography tube. After the yellow filtrate was passed through the resin bed, the tube and the bed were washed with 3 ml of 0.02*N* HCl five times. The colorless effluent and washings were combined and evaporated to dryness in a rotary evaporator under reduced pressure. The dry residue was then dissolved in 3 ml of distilled water and frozen for later use.

For the determination of free amino acids, the samples were brought to room temperature and 0.1 ml of the resulting solution was applied to the chromatographic column for analysis of free amino acids using the Technicon auto amino acid analyzer. Threonine and serine were reported as one figure because the chromatogram peaks for these two amino acids were not clearly separated.

Least-squares analyses (Harvey, 1960) were used to estimate the effect of muscle, sex, age and line of beef cattle on free amino acids. Differences between means of subclasses were tested for significance using the method described by Kramer (1957). The t-distribution as described by Snedecor (1961) was used to test differences in unadjusted means of tough and tender steers. Gross correlation coefficients between free amino acids expressed in micromoles/g of fresh tissue and Warner-Bratzler Shear values were calculated.

RESULTS & DISCUSSION

LEAST-SQUARES means for free amino acids from the l. dorsi and b. femoris muscles and from the Hereford, Angus and inbred Hereford lines of 58 bull carcasses are shown in Table 1. The b. femoris contained significantly greater amounts of glutamic acid, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine and total free amino acids than the l. dorsi after 7 days aging and 4 to 6 months of frozen storage. The b. femoris was significantly ($p < 0.05$) tougher (21.1 lb/in.^{2}) than the l. dorsi (18.2 lb/in.^{2}) when adjacent steaks to those analyzed for free amino acids were tested for tenderness with the Warner-Bratzler Shear. These results are not in agreement with those of Ma et al. (1961) who found that the more tender l. dorsi contained more leucine and isoleucine than the less tender semitendinosus.

It was reported by Tuma et al. (1963) that less tender muscle benefited more from aging than did more tender muscle. Pearson (1963) stated that free amino nitrogen increases as proteolysis or aging proceeds. Zender et al. (1958) and Sharp (1963) have also found that autolysis occurs during aseptic storage of muscle at ambient temperatures. If the tougher b. femoris benefited more from aging 7 days than the l. dorsi, it could have greater amounts of free amino acids because of increased proteolysis.

Muscles from different lines of cattle were not significantly different in tenderness. Nevertheless, the Angus line had significantly greater amounts of valine, methionine, isoleucine, leucine and lysine when compared with the two Hereford lines. The small numbers in this study do not allow a statement about variation in free amino acids due to breed differences. It is possible, however, that the greater amounts of free amino acids in the Angus is related to the fact that they are smaller, earlier maturing cattle than the Herefords.

Usborne (1967) reported that free amino acids, which swine cannot anabolically synthesize, generally increased linearly with weight in muscle tissue. He hypothesized that the increase probably resulted from a decrease in the rate of protein synthesis as the animals matured. In the present study no significant change in free amino acid content of muscles with age was noted. Perhaps it should not have been expected since Field et al. (1966a) found that muscle was still increasing in weight at a faster rate than fat or bone in 20-month-old bulls similar to those in this study. This fact, coupled with a relatively narrow age range of 6 months, partially explains the insignificant change in free amino acid content of muscle with age.

Least-squares means for free amino acids in the l. dorsi muscle of 29 steers and 58 bulls that differed significantly ($p < 0.05$) are given in Table 2. With the exception of threonine and serine, all free amino acids listed were found in more

Table 1—Least-squares means for free amino acids by muscle and line of cattle holding age constant.[1,2]

Free amino acids	Muscle		Line		
	L. dorsi	B. femoris	Herefords	Angus	Inbred Herefords
Threonine & Serine	1.788	1.836	1.674	1.947	1.813
Glutamic acid	0.783[1]	1.145[2]	0.926	1.090	1.052
Proline	0.363	0.291	0.345	0.326	0.310
Glycine	1.167	1.281	1.179	1.168	1.324
Alanine	1.960	2.072	2.169	1.930	1.949
Valine	1.055[1]	1.328[2]	0.993[1]	1.426[2]	1.227[1,2]
Methionine	0.256[1]	0.350[2]	0.286[1,2]	0.353[1]	0.272[2]
Isoleucine	0.416[1]	0.506[2]	0.408[2]	0.521[1]	0.454[1,2]
Leucine	0.740[1]	0.930[2]	0.769[2]	0.909[1]	0.842[1,2]
Tyrosine	0.367[1]	0.442[2]	0.372	0.448	0.393
Phenylalanine	0.345[1]	0.469[2]	0.376	0.452	0.393
Lysine	0.679[1]	0.763[2]	0.634[1]	0.792[2]	0.737[1,2]
Histidine	0.490[1]	0.576[2]	0.489	0.598	0.512
Arginine	0.575	0.627	0.536[2]	0.677[1]	0.612[1,2]
Total free amino acids	10.984[1]	12.616[2]	11.156	12.637	11.890

[1] Means within muscle groups or line of breeding groups bearing different superscript letters differ significantly ($P < 0.05$).

[2] Error degrees of freedom = 111. Least-squares means are expressed in micromoles per gram of fresh muscle.

Table 2—Least-squares means for free amino acids in the l. dorsi muscle by sex.[1,2]

	29 steers	58 bulls
Threonine & serine	1.270[1]	1.765[2]
Methionine	0.374[1]	0.257[2]
Isoleucine	0.492[1]	0.409[2]
Leucine	0.967[1]	0.731[2]
Tyrosine	0.492[1]	0.365[2]
Phenylalanine	0.515[1]	0.342[2]
Lysine	0.786[1]	0.665[2]
Total free amino acids	12.03	11.49

[1] Means bearing different superscript letters differ significantly ($P < 0.05$).

[2] Means are expressed in micromoles per gram of fresh muscle.

abundant amounts in muscles from the steers. Those free amino acids listed in Table 1 and not in Table 2 were not significantly ($p < 0.05$) different in concentration between steers and bulls. Since the steers were choice grade and 20 months of age, greater amounts of free amino acids probably resulted from a decrease in the rate of protein synthesis when compared to the bulls. It is doubtful that proteolysis during aging occurs in the l. dorsi of steers at a faster rate than bulls since bull meat is initially tougher than that from steers (Field et al., 1966b) and would be expected to benefit more from aging.

Warner-Bratzler Shear values were 16.7 and 18.2 lb/in.^{2} for the l. dorsi of the steers and bulls, respectively. Total free amino acids in the steers were slightly higher than in the bulls but this difference was not significant (Table 2). Variability of total free amino acids among species can be noted by comparing the average values of 12.03 and 11.49 micromoles/g of beef muscle in this study with 5.92 micromoles/g of the same free amino acids in pork muscle (Usborne, 1967) and 24.57 or 32.27 micromoles/g respectively in dark or light broiler meat (Miller et al., 1965). The methods used to quantitate free amino acids were similar in the above reports.

The 29 steers were selected as the 14 most tender (12.4 lb/in.^{2}) and the 15 least tender (20.7 lb/in^2) from a group of 109 carcasses that possessed an average Warner-Bratzler Shear value of 16.8 lb/in.^{2} Differences in individual free amino acids in the l. dorsi of the most tender and least tender groups of steers were not significant. Nevertheless, with the exception of valine and histidine, l. dorsi muscles from the more tender steers contained greater amounts of free amino acids than those from the tougher steers.

Within the l. dorsi muscle slightly greater amounts of free amino acids were associated with more tender beef. Therefore, simple correlations between free amino acids and Warner-Bratzler Shear values were obtained. Glutamic acid with a correlation of -0.38 was the only free amino acid in the l. dorsi of steers to be significantly ($p < 0.05$) correlated with Warner-Bratzler Shear values. This agrees with Usborne (1967) who reported that pork tenderness increased as free glutamic acid increased ($r = 0.44$). He also found a significant correlation with leucine and tenderness, not present in this study.

Most other free amino acids studied in the l. dorsi and b. femoris muscles were also negatively correlated with Warner-Bratzler Shear values, but not significantly. Correlating the sum of several different combinations of free amino acids with Warner-Bratzler Shear values did not increase the magnitude of the correlations. When total free amino acids in the l. dorsi were correlated with Warner-Bratzler Shear values of the l. dorsi from 29 steers 20 months old, 34 bulls 14 months old and 24 bulls 20 months old the correlations were -0.23, -0.10 and -0.18, respectively.

These negative correlations suggest that greater quantities of free amino acids are associated with more tender meat. Significant differences in free amino acids due to muscle, sex and line of cattle were found, and greater amounts of free amino acids within muscles were related to tenderness. Nevertheless, simple correlations supporting the hypothesis that greater quantities of free amino acids are associated with more tender meat were not statistically significant for total free amino acids or for most individual free amino acids.

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Some Properties of Mitochondria from Irradiated Tomato Fruit

SUMMARY——Homestead tomato fruit were irradiated with 300 Krad of ^{60}Co gamma rays at three stages of maturity—mature-green, 2 and 6 days from color break. Though oxygen consumption by mitochondrial preparations was greatly suppressed immediately after irradiation it increased to a maximum during the following 2 days and then declined similarily to nonirradiated fruit. Oxygen consumption was reduced considerably during ripening of the nonirradiated and irradiated 6 day breakers. Mitochondria from irradiated fruit exhibited lower P/O ratios than from nonirradiated fruit. A considerable reduction in mitochondrial protein was found during ripening of mature-green fruit. This reduction was less pronounced with the 2 and 6 day breakers. In vitro irradiation of mitochondrial preparations indicated no differences in activity with doses up to 100 Krad, but at 150 and 300 Krad, the activity decreased by 50 and 90%, respectively.

INTRODUCTION

IT IS NOW almost a decade since Burns et al. (1957) reported that respiratory rates of mature green tomatoes increased in proportion to the amount of radiation dose applied. Since then, it has been shown that irradiation enhanced respiration and stimulated ethylene production in both climacteric (Dharkar et al., 1966; Maxie et al., 1966a) and nonclimacteric fruits (Maxie et al., 1965; Ahmed et al., 1966; Dennison et al., 1966; Guerrero et al., 1967; Green et al., 1968).

The maximum increase in respiration occurs during the first 24–48 hr following irradiation, however, the mechanism of this augmented respiratory activity is still obscure. Mitochondria, being the site for respiratory activities, may be best suited to evaluate this mechanism of increased respiration. The purpose of this study was to evaluate some properties of mitochondrial preparations from irradiated tomato fruit.

EXPERIMENTAL

Materials

Tomato fruit (*Lycopersicon esculentum*, Mill, var. Homestead) were irradiated with 300 Krad of ^{60}Co gamma rays at three stages of maturity—mature-green, 2 days and 6 days from color break. The control (0 Krad) and irradiated fruit from each maturity stage were ripened at 20°C and 90% R.H. for 6 days. For in vitro studies, mitochrondria isolated from tomato fruit at the mature-green and 2 day from color break stages of maturity were subjected to radiation doses ranging from 0 to 300 Krad. Fruit were irradiated at 13°C and under continuous flow of air; whereas isolated mitochrondrial preparations were irradiated at 0–4°C.

Mitochrondrial Preparation

The mitochrondria were isolated from control and irradiated fruit every day during the ripening period. The procedure employed was essentially that of Ku et al. (1968) with some modification (Fig. 1, Table 1).

The fruit were washed with distilled water and chilled at 0–4°C in the dark from 1–4 hr before isolation. All subsequent steps were carried out at these temperatures. The chilled fruit were cut into two or more pieces. The seeds and the highly acid locular contents (McCollum, 1956) were carefully and completely removed. The 100 g portions of the outer wall tissue were immediately washed with grinding medium (Table 1) and immersed into the same medium where they were cut into very thin slices with a stainless steel razor blade. Finally, tissue was thoroughly ground with a pestle and mortar.

A pH range of 7.0 to 7.4 was maintained throughout slicing and grinding procedures with dropwise addition of 1 *N* KOH. The homogenate was strained through eight layers of cheesecloth and the remaining procedure is summarized in Figure 1.

Methods

Protein estimation of mitochondrial and soluble fractions were done by the method of Lowry et al. (1951).

Oxygen consumption of the mitochrondrial preparations was measured manometrically (Dickinson et al., 1965) at 30°C with air as the gas phase. Phosphorylation was determined by estimating the disappearance of inorganic phosphorus (Fiske et al., 1925).

Succinic dehydrogenase assay was carried out by measuring the oxygen uptake by Warburg manometer at 30°C (Umbreit et al., 1964). The pH and titratable acidity were determined according to accepted laboratory procedures.

For each determination 100 g of fresh tomato tissue, in duplicate, were obtained from four fruits. Experiments were repeated at least twice and results are reported as mean values.

RESULTS & DISCUSSION

Isolation technique

Several methods were tried for isolation of mitochondria from Homestead tomato fruit (Hulme et al., 1964; Dickinson et al., 1965; Lance et al., 1965; Plesnicar et al., 1967; Ku et al., 1968). Consistent mitochondrial yield and activity were obtained by the use of the method of Ku et al. (1968). Electron microscope studies (Ku et al., 1968) have revealed that their method gives homogeneous and relatively uninjured mitochondria with a higher respiratory control ratio.

Respiration and phosphorylation

Oxygen consumption by tomato mitochondrial preparations was about twice as much when succinate, as compared to malate, was used as a substrate (Fig. 2). The results are in agreement with reported work (Hulme et al., 1964; Ku et al., 1968). Oxygen consumption by mitochondrial preparations was greatly suppressed immediately after irradiation, but increased to a maximum during the fol-

Table 1—Composition of media.

Grinding	Washing	Suspending
pH, 7.6	pH, 7.2	pH, 7.0
Sucrose, 0.4 *M*	Sucrose, 0.4 *M*	Mannitol, 0.3 *M*
Cysteine, 4 m*M*	KH_2PO_4, 10 m*M*	Tris-HCL, 0.01 *M*
$MgCl_2$, 2 m*M*	KCL, 10 m*M*	$MgCl_2$, 0.005 *M*
KCL, 10 m*M*	Bovine serum	
EDTA, 10 m*M*	albumin 0.5 mg/ml	
Tris-HCL, 50 m*M*		
Bovine serum		
albumin 0.5 mg/ml		

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lowing 2 days and then declined similarly to the nonirradiated fruit (Fig. 2). Oxygen uptake was reduced considerably during ripening of the nonirradiated and irradiated 6 day breakers.

The increase in mitochondrial activity to a maximum is in accord with the reported carbon dioxide evolution from peaches and nectarines (Maxie et al., 1966b) and from lemons and oranges (Maxie et al., 1965; Ahmed et al., 1966; Dennison et al., 1966; Green et al., 1968). Tomato fruit irradiated with doses of 0.5 $\times$ 10^6 — 1.5 $\times$ 10^6 rep cathode rays exhibited an initial increase in CO_2 evolution followed by a decrease and then an increase to a maximum in 96 hr which was directly proportional to the dose supplied (Burns et al., 1957).

The considerable suppression in mitochondrial activity immediately after irradiation followed by increased activity to a maximum (Fig. 2) might be due to a repair phenomenon of a radiation damage. Probably, mitochondrial preparations isolated immediately after irradiation were more liable to damage during the isolation procedure than those isolated from nonirradiated fruit.

Irradiation might also have induced an immediate change in permeability of mitochondrial membrane which ultimately restricted or inhibited incoming supply of substrates to mitochondria. The permeability seemed to be restored through subsequent ripening periods.

Ethylene was shown to change the permeability of isolated mitochondria (Lyons et al., 1964). Maxie et al. (1965, 1966a) reported that ethylene production was stimulated immediately following irradiation. The production of ethylene or any other hydrocarbon stimulated by irradiation might have ultimately resulted in suppression of activity.

Cytoplasmic particles isolated from pear fruit exhibited suppressed oxygen uptake immediately after irradiation followed by increased activity for 6 days (Miller et al., 1964). Diminution in density of the intracellular particles with tissue aging was also found. However, Romani et al. (1964) reported a sharp fall in respiration of mitochondrial suspensions from fruits irradiated with 1000 Krad or more.

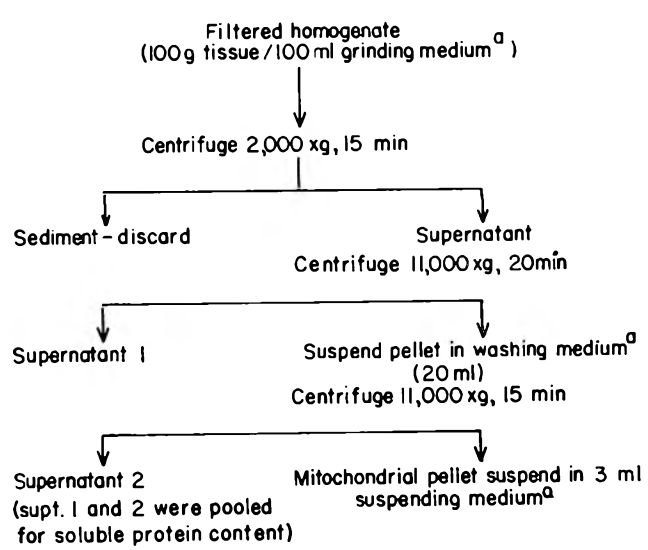
Accelerated oxygen uptake using succinate as a substrate also concomitantly increased the rate of phosphorylation or formation of ATP. This has helped to maintain P/O ratios similar to those when malate was used as a substrate (Table 2). However, mitochondrial preparations from irradiated fruit exhibited lower P/O ratios than those from nonirradiated fruit indicating a slight reduction in phosphorylation.

Table 2—P/O in tomato mitochondria isolated from control and irradiated fruit.

Days (Post-irradiation)	Substrate: Malate, Dose (Krad) 0	Malate, 300	Succinate, 0	Succinate, 300
Mature-green				
0	2.31	1.98	2.35	2.18
2	2.28	2.31	2.40	2.37
4	2.41	2.00	2.45	2.25
6	2.31	1.85	2.35	2.15
2 day breakers				
0	2.45	2.00	2.30	2.10
2	2.40	2.29	2.25	2.30
4	2.31	2.11	2.17	2.25
6	2.55	2.00	2.11	2.00
6 day breakers				
0	2.00	1.73	2.00	1.95
2	1.98	1.80	2.18	2.10
4	2.10	1.65	1.98	1.85
6	1.80	1.63	2.00	1.78

Succinic-dehydrogenase activity of mitochrondrial suspension

A decline in membrane permeability or dehydrogenase activity during ripening could account for the lower oxidative ac-



a For medio composition see Table 1.

Fig. 1—Isolation of mitochondria from tomato fruit.

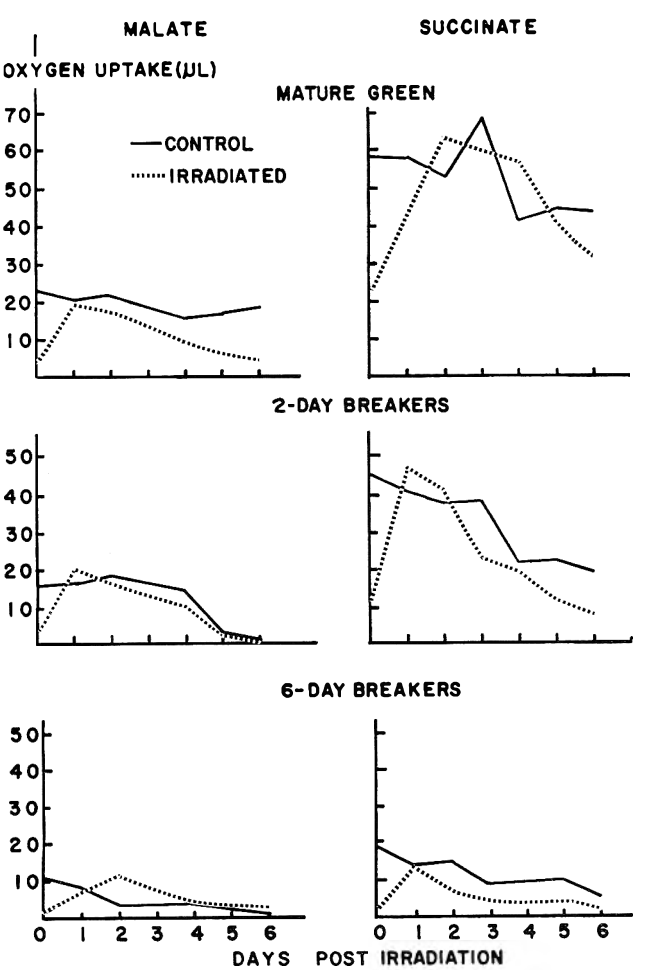


Fig. 2—Oxygen uptake by mitochondrial preparations using malate and succinate as subtsrates, from control and irradiated tomato fruit at mature-green, 2 days and 6 days from color breaks.

Table 3—Succinic dehydrogenase activity[1] in tomato mitochondria isolated from control and irradiated fruit.

Days (Post-irradiation)	Mature-green		2 day breakers		6 day breakers	
	Dose (Krad)					
	0	300	0	300	0	300
0	61.5	43.7	62.5	41.8	49.4	38.2
1	64.3	58.2	62.0	59.0	50.5	47.2
2	69.3	69.9	58.3	55.3	48.6	45.1
3	71.5	70.4	57.1	54.8	45.2	43.3
4	70.3	71.6	55.0	54.7	39.5	37.2
5	66.5	70.1	53.0	52.9	35.2	35.1
6	63.2	68.3	55.5	47.8	38.3	33.0

[1] μl O_2 uptake/hr/100g fresh tissue.

Table 4—Changes in mitochondrial and soluble protein[1] during ripening of control and irradiated tomato fruit.

Days (Post-irradiation)	Mitochondria		Soluble	
	Dose (Krad)			
	0	300	0	300
Mature green				
0	16.3	17.5	25.6	25.9
2	16.9	17.5	24.9	25.6
4	12.5	11.9	25.6	25.0
6	10.3	10.2	25.6	26.4
2 day breakers				
0	9.4	9.4	24.7	23.8
2	9.4	8.8	25.0	24.7
4	8.2	8.9	24.1	25.6
6	7.5	9.0	25.9	26.5
6 day breakers				
0	8.3	8.1	21.9	22.2
2	8.0	8.1	22.2	21.9
4	8.2	8.1	21.8	23.6
6	7.5	8.0	22.8	23.8

[1] mg protein/100g fresh tissue.

Table 5—Changes in pH and titratable acidity (ml 0.1N NaOH) during ripening of control and irradiated tomato fruit.

Days (Post-irradiation)	pH		Acidity	
	Dose (Krad)			
	0	300	0	300
Mature green				
0	4.45	4.35	5.90	5.95
1	4.45	4.25	6.20	6.55
2	4.35	4.65	6.60	5.30
3	4.25	4.55	7.55	5.60
4	4.25	4.65	7.80	5.30
5	4.40	4.80	5.35	4.95
6	4.20	4.85	6.90	4.75
2 day breakers				
0	4.40	4.40	5.50	5.50
1	4.65	4.30	5.60	6.90
2	4.35	4.55	5.70	4.80
3	4.40	4.65	5.45	4.70
4	4.45	4.55	6.10	4.65
5	4.55	4.65	5.50	4.75
6	4.50	4.60	5.20	4.50
6 day breakers				
0	4.50	4.45	5.20	5.40
1	4.45	4.40	6.10	5.60
2	4.70	4.60	4.90	4.50
3	4.55	4.65	5.10	4.90
4	4.60	4.65	5.00	4.50
5	4.65	4.70	4.90	4.30
6	4.65	4.65	4.95	4.55

tivity of mitochondrial preparation of ripe tomato fruit (Dickinson et al., 1965). Irradiation may alter membrane permeability or dehydrogenase activity. Succinic-dehydrogenase activity accordingly showed a significant drop following irradiation treatment (Table 3). The loss in the activity of this enzyme could explain the suppression of oxygen uptake by mitochondrial preparations immediately after irradiation. This suppression seems to be restored as the ripening period was prolonged. However, the overall trend of succinic-dehydrogenase activities from mature-green to 2 day and 6 day breaker stages resemble the climacteric pattern of the fruit.

Yield of soluble and mitochrondrial protein

During ripening of the mature-green fruit, yield of mitochondrial protein declined more rapidly than the yield of soluble protein (Table 4). The results indicate degradation of the particulate fraction of the cells during ripening. Similar trends were observed for the irradiated fruit. A decrease in overall particulate yield was noted following irradiation of fruit tissues (Romani et al., 1962). The apparent loss of intracellular structures was followed by a partial recovery after low doses (250–300 Krad), but not after high doses (600–1000 Krad) (Romani et al., 1962). The change in particulate material may be a function of fruit age. The results also suggest the overall reduction in protein synthesis as the fruit approaches senescence.

pH and total acidity

The obtained values for pH and acidity in both control and irradiated fruit were less indicative of any significant role pertaining to mitochondrial activity in the present study. However, a drop in pH values was observed 24 hr following irradiation which was concurrent with the increase in respiratory activity (Table 5). Production of some types and amounts of organic acids may have been stimulated or inhibited resulting in the varied amounts of titratable acidities (Table 5).

Hulme (1959) noted that succinic acid accumulated in irradiated fruit; whereas Maxie et al. (1964) showed a marked decrease in total acidity (expressed as percent citric acid) in irradiated lemon fruit. Romani (1964) presented data indicating that the loss in acidity is not a direct radio-decarboxylation of acids. The amount of ascorbic acid in tomatoes within each ripeness class declined with increasing dose and time (Maxie et al., 1966b).

Table 6—In vitro irradiation of mitochondrial preparation: respiration[1] and phosphorylation.[2]

Dose (Krad)	Mature-green		2 day breakers	
	O_2 uptake	P/O	O_2 uptake	P/O
0	57.2	2.35	45.1	2.11
50	56.2	2.21	43.8	2.15
100	59.5	2.45	46.3	2.10
150	31.0	1.95	23.2	2.35
200	10.2	2.00	7.0	2.10
300	6.6	2.10	3.0	1.95

[1] μl O_2 uptake/hr/10 g fresh tissue, using Na-succinate as substrate.
[2] P/O ratio.

In vitro irradiation of mitochrondria

Isolated mitochondrial suspensions from tomato fruit (mature-green and 2 day breakers) were subjected to different doses of radiation (0–300 Krad). The respiration and phosphorylation rates varied from those obtained in the in vivo studies. It seems that up to 100 Krad, irradiation did not influence the activity, but there was about 50 and 90% reduction in oxygen consumption at 150 and 300 Krad, respectively (Table 6). As the rate of phosphorylation was decreased simultaneously with the increase in radiation doses, P/O ratios remained unchanged. Mitochondria from mature-green fruit exhibited higher rates of oxygen consumption.

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The Volatile Components of Irradiated Beef and Pork Fats

SUMMARY—Samples of beef and pork fats were irradiated under vacuum at seven doses ranging from 0.5 to 6.0 megarads. Of the components produced by irradiation, 41 were identified by gas chromatography and mass spectrometry. These include a series of n-alkanes, 1-alkenes, internally unsaturated alkenes and alkadienes. The latter two groups of compounds have not been reported previously in meats or meat fats. In addition, hexadecanal, octadecanal and octadecenal were found to be produced in relatively large quantities by irradiation. Quantitative data showed that a definite relationship exists between the volatile pattern and the fatty acid composition of the irradiated material. Flavor threshold values of the unsaturated classes of hydrocarbons vary widely among themselves but are much lower than those of the n-alkanes.

INTRODUCTION

THE MAJOR obstacle to practical application of high-energy radiation in the preservation of meats has been the development of off-odors that render the product unacceptable. In search of the causes of flavor deterioration some investigators attempted to isolate and identify the volatiles from irradiated meats (Batzer et al., 1955, 1957; Burks et al., 1959; Merritt et al., 1959; Monty et al., 1961; Salvador et al., 1962; Wick et al., 1961) while other workers studied the effects of irradiation on meat components such as fats and proteins (Chipault et al., 1966; Hedin et al., 1961; Merritt et al., 1966; Rhodes et al., 1964; Sribney et al., 1955; Witting et al., 1958).

Amines, sulfur compounds and some carbonyl compounds were presumed to originate from the meat proteins while the lipid fraction is believed to produce carbonyl compounds and hydrocarbons. In some cases the identified compounds did not differ qualitatively from those found in unirradiated samples and in other instances they were typical of the classic products of fat oxidation.

More recently, Merritt et al. (1966) irradiated beef, veal, mutton, lamb and pork meats and identified the normal alkanes from C_1 to C_{15}, the alkenes from C_2 to C_{14} and the C_{10} and C_{11} alkynes. With the exception of undecyne, these compounds were found in all five samples in similar quantities. No aldehydes higher than C_8 were detected. The authors concluded that the hydrocarbons originated from the meat lipids and that their formation resulted from random splitting at carbon-carbon bonds along the fatty acid chains, and emphasized their possible role in the development of irradiation off-odor.

On the other hand, Wick et al. (1963, 1965a,b), who irradiated enzyme-deactivated beef in the presence of air, attributed the off-odor to carbonyl compounds. These workers, however, also identified n-alkanes and alkenes up to C_{16} in irradiated beef.

In the present study, beef and pork fats were irradiated under non-oxidative conditions and the volatile components studied qualitatively and quantitatively. The effect of irradiation dose on the formation of hydrocarbons and the flavor significance of the latter compounds are reported.

EXPERIMENTAL

Materials

Beef and pork fats were selected from

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the perinephric and the outer loin portions respectively. The fats were melted in an open steam-jacketed kettle and then filtered through eight layers of cheesecloth. Fifteen-g samples were sealed under vacuum in either 30 ml ampoules or in special break-seal flasks (125 ml) similar to those described by Merritt et al. (1966).

Saturated and unsaturated hydrocarbons (99% purity) were purchased from Polyscience Laboratories, Evanston, Ill. These compounds were checked for purity by flame ionization gas chromatography. When necessary, the hydrocarbons were purified by preparatory gas chromatography.

Irradiation

The samples were transported by private automobile to the U.S. Army Laboratories in Natick, Massachusetts, a distance of approximately 70 miles. Irradiation was performed at controlled temperatures using the 1.1 megacurie cobalt 60 source. Immediately after irradiation, the samples were packed in dry ice and returned to the laboratory for analysis.

Collection of volatiles

The irradiated samples and the unirradiated controls were distilled under identical conditions. For analysis of the higher boiling compounds (C_9–C_{17}), samples were transferred from the ampoules to appropriate distillation flasks, distilled under vacuum (10^{-3} mm) at 80 C for 2 hr and the volatiles collected in a liquid nitrogen vacuum trap (Corning 7729). For the low boiling compounds the radiation flasks fitted with the break-seal tubes were connected to a 4 × ⅛-in precolumn packed with 60/80 mesh F-20 activated alumina, which was in turn connected to a high vacuum pump.

The precolumn was held in liquid nitrogen and the volatile collection was initiated by shattering the break-seal with a magnetic bar and continued for 2 hr after which the precolumn was fitted between the injection port of the gas chromatograph and the inlet of a 12-ft alumina column. The liquid nitrogen was then removed and the carrier gas flow resumed for GLC analysis.

Gas chromatography (GC)

Three gas chromatographic instruments were used in this study: an F & M model 810, an Aerograph model 1200 and a Perkin-Elmer model 226—all equipped with flame ionization detection systems. The higher-boiling compounds were separated on two 12-ft × ⅛-in. columns; one packed with 10% silicone rubber SE-30 and the other with 15% Carbowax 20 M. Both columns were programmed from 60–200°C at 2°/min. A 200-ft × 0.02-in. capillary column was used in the Perkin-Elmer instrument.

The lower-boiling compounds were separated on a 12-ft × ⅛-in. column packed with activated alumina F-20 with programming at 15°/min from 60° to 360°C. Analysis of hydrocarbons by gas-solid chromatography was described earlier by List et al. (1965).

Methyl esters of fatty acids were analyzed on a 12-ft × ⅛-in. column packed with diethylene glycol succinate.

Table 1—Fatty acid composition of beef and pork fats used in this study (%).

Fatty Acid	Beef	Pork
Myristic	4.1	1.6
Palmitic	27.9	20.8
Palmitoleic	2.9	5.1
Stearic	31.4	10.6
Oleic	33.4	60.6
Linoleic	0.3	1.3

The hydrocarbons used for flavor threshold determination were purified by preparative gas chromatography using an 8-ft × ½-in. column packed with silicone SE-30 in an F & M model 720 gas chromatograph equipped with a thermal conductivity detector.

Mass spectrometry

A combination GC-mass spectrometer system was used, in which 50% of the column effluent was directed to the flame ionization detector while the other 50% was admitted via a heated line to a Biemann helium separator and then to the ion source of a Hitachi-Perkin-Elmer RMU-6A mass spectrometer. The source was operated at 80 electron volts and the ionization chamber held at 250°C. The electron multiplier was operated at 3,000 volts and a sensitivity of 1 or at 2,500 volts and a sensitivity of 10, depending on the size of the GC peak. The ion source was equipped with a total ion monitor, the output of which was amplified by a Keathly piccoammeter and displayed on a strip chart recorder. Spectra were recorded with a Honeywell 1508 Visicorder.

Quantitative analysis

The concentration of each radiolytic product in the fat was determined by the use of an appropriate internal standard and conversion factors as described previously by Buziassy et al. (1968). A hydrocarbon,

Table 2—Identification of the volatiles formed in beef and pork fats by irradiation.

	GC retention			Mass spectrometry	
	Agreement with auth. compds. on:				
Compound	Alumina	Carbowax	Silicone	Agreement with auth. compds.	Interpretation only
Alkanes:					
Methane	X				
Ethane	X				
Propane	X			X	
Butane	X			X	
Pentane	X			X	
Hexane	X			X	
Heptane	X		X	X	
Octane	X	X	X	X	
Nonane	X	X	X	X	
Decane	X	X	X	X	
Undecane		X	X	X	
Dodecane		X	X	X	
Tridecane		X	X	X	
Tetradecane		X	X	X	
Pentadecane		X	X	X	
Hexadecane		X	X	X	
Heptadecane		X	X	X	
1-Alkenes:					
Ethylene	X				
Propene	X				X
Butene	X			X	
Pentene	X			X	
Hexene	X			X	
Heptene	X		X	X	
Octene	X	X	X	X	
Nonene	X	X	X	X	
Decene	X	X	X	X	
Undecene		X	X	X	
Dodecene		X	X	X	
Tridecene		X	X	X	
Tetradecene		X	X	X	
Pentadecene		X	X	X	
Hexadecene		X	X	X	
Heptadecene		X	X	X	
Internal Alkenes:					
Pentadecene		X	X		X
Hexadecene		X	X		X
Heptadecene		X	X		X
Alkadienes:					
Tridecadiene		X	X		X
Tetradecadiene		X	X		X
Pentadecadiene		X	X		X
Hexadecadiene		X	X		X
Heptadecadiene		X	X	X	
Aldehydes:					
Hexadecanal				X	
Octadecanal				X	
Octadecenal				X	

6-dodecyne, which is not produced by irradiation does not interfere with the GC peaks of the irradiated pork or beef fats, was used. Equal amounts of the n-alkane and 1-alkene series from C_9 to C_{19} plus the internal standard were added to fresh pork fat and the volatiles analyzed under the conditions described above.

A conversion factor for each compound was calculated from the ratio of the GC peaks of the internal standard to that of the compound. An accurately weighed amount of the internal standard was added to each sample immediately before distillation and the amount of each compound in the fat was calculated according to the formula:

$$\text{mg Hc in sample} = \frac{\text{PHc} \times C_{Hc} \times \text{Is}}{P_{Is}}$$

where Hc = hydrocarbon
PHc = peak area of hydrocarbon
C_{Hc} = conversion factor for hydrocarbon
Is = mg internal standard added
P_{Is} = peak area of internal standard

The amounts of the lower-boiling compounds were determined by means of a number of standard curves obtained from the analysis of a series of varying quantities of the shorter-chain hydrocarbons on alumina as described above.

Odor threshold determinations

The methods and procedures for threshold determinations were reviewed by Guilford (1954), and the precautions suggested therein were observed throughout this investigation.

A panel of 8 to 10 members was chosen on the basis of consistency, and odor evaluations were conducted in a special taste panel laboratory. Six different concentrations of each compound in mineral oil were presented at random in each session. Panel members were asked to smell each sample and report whether or not it contained a chemical. A control sample of mineral oil was supplied to the panel for comparison.

The individual's odor threshold was considered to be the lowest consistently correct concentration. The odor threshold of each hydrocarbon was determined by averaging the entire panel's scores. The thresholds were expressed in mg/100 g of mineral oil.

RESULTS & DISCUSSION

THE fatty acid composition of the beef and pork fats used in this study is given in Table 1. While the C_{16}, C_{18} and $C_{18:1}$ acids were present in approximately equal amounts in the beef fat, oleic acid accounted for more than half of the pork fatty acids.

Table 2 shows the compounds identified in irradiated beef and pork fats and the criteria used for their identification. These compounds were found in both irradiated fats but were not detected in the unirradiated controls under the experimental conditions used.

The alkanes and 1-alkenes gave mass

Table 3—Quantitative analyses of the volatiles formed in beef fat by irradiation at 25°C (mg/100 g).

Compound	Megarads						
	0.5	1	2	3	4	5	6
n-propane	0.040		0.082		0.168		0.298
1-propene	0.007		0.022		0.021		0.026
n-butane	0.034		0.088		0.140		0.252
1-butene	0.018		0.016		0.016		0.017
n-pentane	0.050		0.089		0.114		0.223
1-pentene	0.002		0.004		0.007		0.015
n-hexane	0.006		0.028		0.086		0.174
1-hexene	0.001		0.005		0.029		0.036
n-heptane	0.026	0.058	0.104	0.172	0.220	0.284	0.402
1-heptene	0.007	0.022	0.038		0.065	0.068	0.114
n-octane	0.030	0.071	0.172	0.176	0.244	0.351	0.530
1-octene	0.005	0.029	0.050	0.056	0.075	0.081	0.154
n-nonane	0.081	0.054	0.096	0.148	0.199	0.276	0.374
1-nonene	0.012	0.028	0.039	0.049	0.074	0.083	0.101
n-decane	0.049	0.069	0.127	0.166	0.230	0.311	0.439
1-decene	0.022	0.038	0.070	0.091	0.124	0.160	0.192
n-undecane	0.040	0.062	0.108	0.132	0.197	0.257	0.326
1-undecene	0.016	0.023	0.054	0.067	0.088	0.100	0.130
n-dodecane	0.037	0.053	0.087	0.122	0.167	0.235	0.296
1-dodecene	0.067	0.108	0.174	0.249	0.361	0.462	0.618
n-tridecane	0.095	0.160	0.293	0.379	0.602	0.827	1.131
1-tridecane	0.067	0.087	0.141	0.151	0.312	0.324	0.438
tridecadiene	trace	trace	trace	trace	trace	trace	trace
n-tetradecane	0.055	0.090	0.131	0.158	0.253	0.337	0.475
1-tetradecene	0.423	0.643	1.40	1.54	2.42	3.11	3.78
tetradecadiene	0.026	0.050	0.086	0.122	0.166	0.202	0.307
n-pentadecane	0.285	0.504	1.04	1.48	2.45	2.94	3.625
pentadecene (int.)[1]	0.048	0.048	0.101	0.16	0.211	0.259	0.388
1-pentadecene	0.064	0.097	0.195	0.242	0.350	0.428	0.729
pentadecadiene	0.026	0.041	0.073	0.106	0.168	0.193	0.267
n-hexadecane	0.060	0.078	0.102	0.096	0.182	0.230	0.450
hexadecene (int.)[1]	trace	trace	trace	trace	trace	trace	trace
1-hexadecene	0.418	0.584	0.778	1.436	2.35	2.77	3.105
hexadecadiene	0.564	0.739	1.26	1.854	2.91	3.52	4.04
n-heptadecane	0.418	0.500	0.822	1.255	2.14	2.19	2.69
heptadecene (int.)[1]	0.378	0.544	0.594	1.562	2.59	2.70	3.29
1-heptadecene	trace	0.112	0.172	0.222	0.198	0.432	0.51
heptadecadiene	0.096	0.133	0.263	0.310	0.407	0.635	0.82

[1] Internally unsaturated.

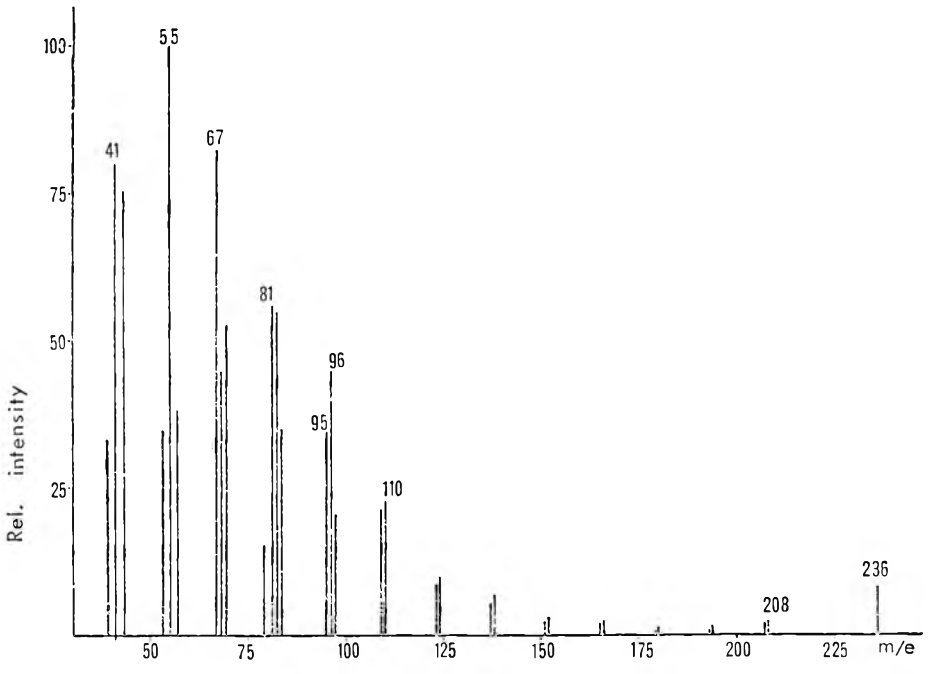

Fig. 1—Mass Spectrum of 1,8-heptadecadiene.

Table 4—Quantitative analyses of the volatiles formed in pork fat by irradiation at 25°C (mg/100 g).

Compounds	Megarads						
	0.5	1	2	3	4	5	6
n-propane	0.034		0.091		0.215		0.370
1-propene	0.020		0.037		0.096		0.133
n-butane	0.029		0.080		0.182		0.307
1-butene	0.004		0.035		0.058		0.079
n-pentane	0.029		0.068		0.140		0.280
1-pentene	0.004		0.024		0.042		0.068
n-hexane	0.007		0.041		0.107		0.193
1-hexene	0.012		0.033		0.084		0.204
n-heptane	0.042	0.031	0.069	0.121	0.259	0.302	0.351
1-heptene	0.019	0.028	0.064	0.106	0.178	0.250	0.290
n-octane	0.071	0.066	0.114	0.172	0.198	0.322	0.480
1-octene	0.029	0.044	0.068	0.096	0.176	0.220	0.258
n-nonane	0.016	0.036	0.076	0.123	0.202	0.236	0.254
1-nonene	0.021	0.046	0.082	0.122	0.138	0.223	0.301
n-decane	0.021	0.044	0.097	0.134	0.160	0.226	0.270
1-decene	0.044	0.090	0.170	0.243	0.325	0.437	0.520
n-undecane	0.035	0.047	0.086	0.114	0.144	0.199	0.278
1-undecene	0.024	0.041	0.073	0.083	0.102	0.140	0.166
n-dodecane	0.035	0.045	0.037	0.116	0.127	0.162	0.200
1-dodecene	0.052	0.089	0.183	0.287	0.314	0.460	0.515
n-tridecane	0.047	0.079	0.140	0.194	0.242	0.315	0.447
1-tridecene	0.025	0.050	0.089	0.142	0.196	0.227	0.334
tridecadiene	trace	trace	trace	trace	trace	trace	trace
n-tetradecane	0.021	0.039	0.078	0.088	0.144	0.164	0.204
1-tetradecene	0.188	0.402	0.794	1.24	1.75	2.03	2.80
tetradecadiene	0.044	0.084	0.158	0.276	0.383	0.408	0.579
n-pentadecane	0.196	0.335	0.662	0.978	1.39	1.80	2.10
pentadecene (int.)[1]	0.036	0.063	0.113	0.206	0.274	0.330	0.344
1-pentadecene	0.037	0.059	0.166	0.208	0.332	0.334	0.508
pentadecadiene	0.040	0.054	0.074	0.141	0.191	0.212	0.258
n-hexadecane	0.012	0.017	0.003	0.059	0.066	0.084	0.108
hexadecene (int.)[1]	0.018	0.018	0.032	0.050	0.097	0.108	0.151
1-hexadecene	0.101	0.162	0.329	0.462	0.639	0.840	0.945
hexadecadiene	0.486	1.02	1.97	2.67	3.81	4.90	6.84
n-heptadecane	0.077	0.156	0.280	0.579	0.727	0.805	0.984
heptadecene (int.)[1]	0.317	0.690	1.41	1.90	2.99	3.58	3.96
1-heptadecene	trace	trace	trace	trace	trace	trace	trace
heptadecadiene	trace	0.215	0.420	0.630	1.03	1.08	1.28

[1] Internally unsaturated.

spectra and GLC retention times identical to those of authentic compounds. The mass spectra of the alkenes with internally situated double bond was very similar to the 1-alkenes of the same carbon numbers but their GLC retention times were different. On Carbowax columns, they emerged after the corresponding *n*-alkanes but before the corresponding 1-alkenes.

While this order of elution is expected on the basis of polarity differences, additional evidence concerning position of unsaturation was obtained on the basis of differences in mass spectral peak intensities. Under mass spectrometric conditions, cleavage at positions beta to the double bond is more frequent than rupture at other sites along the chain (Reed, 1965). Examination of the mass spectra of the two successive GC peaks of pentadecene, for example, showed that the fragment at m/e 153 was approximately 30% less abundant in the slower peak, which coincided with the 1-alkene. This indicated more abundant splitting between carbons 4 and 5, which in turn suggested that the double bond was in the 6 position.

The series of hydrocarbons eluting on Carbowax columns after the 1-alkenes were different in their retention characteristics from alkynes but gave mass spectra typical of the compounds with the general formula C_nH_{2n-2}. More specifically, the spectra included the common fragment ion series: 39, 53, 67, 81, 95, 109, 123 . . ., etc., as well as the typical peaks at even numbered m/e. Further evidence that they were dienes was obtained by the fact that the GLC peak assigned the structure of heptadecadiene agreed in retention time with, and gave a mass spectrum identical to, that of an authentic sample of 1,8-heptadecadiene (Fig. 1).

Gas chromatographic peaks identified as aldehydes agreed in retention with the extrapolated values of the available lower members of the series (C_{10}-C_{14}) and gave mass spectra similar to those of reference spectra of authentic compounds.

The observation that hydrocarbons are the major radiolytic products in fats is in agreement with the findings of Merritt et al. (1966). It should be pointed out, however, that the alkadienes and some of the longer chain alkanes and alkenes identified in the present study, have never been reported previously in meats or meat fats.

Quantitative data for the volatiles formed in beef and pork fats irradiated at seven different dosages are presented in Tables 3 and 4 respectively. The hydrocarbons are listed in order of increasing elution time on Carbowax columns. It can be seen that the amounts of the various hydrocarbons formed by irradiation vary widely. In both fats six hydrocarbons (i.e., 1-tetradecene, pentadecane, 1-hexadecene, hexadecadiene, heptadecane and heptadecene) were produced in large quantities as compared to the shorter-chain hydrocarbons.

The major hydrocarbons possessed either one or two carbon atoms less than the major fatty acids present in the fats studied. Furthermore, beef fat produced approximately 3 and 4 times as much of 1-hexadecene and *n*-heptadecane respectively as did pork fat. Interestingly, the beef fat used in this study contained almost three times as much stearic acid as pork fat. No attempt was made to measure the aldehydes quantitatively. The size of their peaks, however, indicates that they were formed in relatively large quantities.

These results suggested a relationship between fatty acid composition and the major radiolytic products and agreed with work simultaneously carried out in this laboratory by Dubravcic et al. on the radiolysis of fish oil (1968a) and simple triglycerides (1968b). As revealed in the latter study, 1-tetradecene and n-pentadecane were the major hydrocarbons from palmitic acid, 1-hexadecene and *n*-heptadecane from stearic and hexadecadiene and heptadecene from oleic.

It is clear that radiolytic splitting of fatty acid chains is not random but follows a preferential pattern resulting in an uneven distribution of the hydrocarbons formed.

The effect of irradiation dose on the formation of the various hydrocarbons is shown in Tables 3 and 4. The increased production of volatiles with increase in dose has been previously observed by Merritt et al. (1959). It is interesting to note, however, that the rate of formation (amount of compound produced per megarad) for the six major hydrocarbons mentioned above is much greater than that of the shorter-chain compounds.

Flavor significance of hydrocarbons

Beef and pork fats developed a typical off-odor upon irradiation. This odor was detectable at 2 megarads and was more intense at the higher dosages. Merritt and co-workers implicated hydrocarbons in the off-odor of irradiated fats. The presence of hydrocarbons in oxidized fats was

Table 5—Odor thresholds of some aliphatic hydrocarbons.

Carbon no.	mg/100 g mineral oil			
	n-alkanes	1-alkenes	1-alkynes	alkadienes
5	34.	9.2	18.	
6	9.	0.3	16.	0.8
7	25.	0.1	10.	0.5
8	134.	0.2	0.8	1.0
9	215.	2.6	1.8	2.9
10	400.	15.	6.0	4.6
11	575.	57.	11.	
12	>1300.	128.		
14	>1300.	168.		
15	>1300.	225.		
16	>1300.	320.		

reported by Buttery (1961); Day et al., (1963); Merritt et al., (1967); and Krishnamurthy et al. (1967).

To determine the organoleptic significance of hydrocarbons, flavor threshold evaluations were conducted with four classes of these compounds. As shown in Table 5, the unsaturated hydrocarbons are much more odorous than the saturated ones. The 1-alkenes contain the most odorous compounds: hexene, heptene and octene.

The values presented in Table 5 provide the potential for a practical application. For example, if certain compounds were known to exhibit unpleasant odors, it would be possible, by consulting odor threshold data showing relationships between dosage and the amounts formed by irradiation, to predict how high a dose can be applied to a product before such compounds make their presence felt by the consumer.

The quantities of 1-heptene and 1-octene, in both beef and pork fats irradiated at 6.0 megarads, approached or exceeded their odor threshold. The threshold of 1-hexene was approached in pork fat irradiated at the same dosage. On the other hand, the concentrations of the other hydrocarbons were well below their thresholds. Since the fats irradiated at 2 megarads exhibited some off-odor and since at this dose, all hydrocarbons were below their threshold, it may be possible that additive or synergistic effects are involved.

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Identification and Characterization of the Pectic Enzymes of the McFarlin Cranberry

SUMMARY—*Endo-polygalacturonase activity was found in cranberry proteins. Viscosity measurements showed that the hydrolysis of pectins with both high and low methoxyl content occurred on addition of a protein dialysate to pectin solutions. The use of a phenol binding agent in the enzyme preparation was necessary to obtain a high hydrolytic activity. Higher activity was noted with citrus pectins than cranberry pectins. Optimum activity was found at pH 5.0 for cranberry polygalacturonase. Activity was destroyed after 35 min of heating at 100°C. Up to 0.6 M NaCl gave no significant effect on cranberry polygalacturonase activity at pH 5.0. Cranberry pectin esterase activity was low when compared to strawberry and tomato pectin esterase. The optimum pH value for pectin esterase was 7.5, and the enzyme was inactivated when heated for 5 min at 100°C. When pectin solutions were treated with NaCl, optimum pectin esterase activity occurred at 0.15 M concentration.*

INTRODUCTION

THE PRESENCE of pectic substances in the plant cell plays an important role in maintaining rigidity to plant tissues and contributes to the consistency of fruit purees and juices. Pectic enzymes act on the pectin polymer to produce smaller fragments, thus disrupting the rigidity of the tissue and affecting the consistency of the juice or puree.

The enzymes involved are polygalacturonase, pectin esterase, and pectin transeliminase. They may be inherent in the plant tissue or may be produced by different types of microorganisms.

The gelling power of cranberry lies in its high content of pectin, facilitating the production of cranberry sauce and jelly. Pectic enzymes in cranberry could increase the rate of hydrolysis of the pectins present before heating, thus affecting the quality of the product.

Therefore, pectic enzymes of the McFarlin cranberries were extracted by different methods and hydrolytic activities were measured at different pH's and salt concentrations.

EXPERIMENTAL

PHENOLIC COMPOUNDS, namely tannins, are abundant in the mature cranberry and upon maceration of the berries for enzyme extraction, these compounds may bind irreversibly with proteins by hydrogen bonding on oxidation of tannins. Therefore, enzyme activity is greatly retarded. For this reason, phenols have been removed from the enzymes preparation by polyethylene glycol (Badran and Jones, 1965) and polyvinylpyrrolidone (Loomis et al., 1966).

Source of the fruit

The McFarlin cranberries, *Vaccinium macrocarpon*, used for this study were grown near Markham, Washington. The ripe harvested berries were placed in polyethylene bags, sealed in tin containers, frozen and stored at a temperature of −20°C.

Preparation of acetone powder

With all equipment pre-cooled and maintained at −20°C, 100 g of washed frozen cranberries were blended for 3 min with 5 volumes (v/v) of acetone (−20°C). The slurry was filtered by suction through Whatman No. 1 filter paper. The solid material was washed several times with 200 ml portions of cold acetone and dried for 20 hr at 25°C over concentrated H_2SO_4 under vacuum. The acetone powder was stored in a tight flask at −20°C. The yield was 4.3 g of powder per 100 g of frozen tissue.

Preparation of acetone powder in presence of polyethylene glycol (PEG)

The method of Badran et al (1965) was modified as follows: 100 g of frozen cranberries were added to 100 ml of 2% polyethylene glycol. Cold acetone (−20°C) was also added in the ratio of 1/5 (suspension/acetone, v/v). The mixture was blended in a Waring Blendor for 3 min at full speed. The suspension was filtered by suction through Whatman No. 1 filter paper. The cake was washed, dried, and stored as above.

The method employed for the preparation of cranberry proteins in presence of polyvinylpyrrolidone (PVP) was the modified procedure developed by Loomis et al. (1966).

Precipitation and dialysis of cranberry proteins

Four g of the acetone powder preparation were suspended in 800 ml of cold 0.15 *M* NaCl solution at pH 7.5 and stirred gently for 30 min. The suspension was squeezed through a nylon cloth followed by filtration through Whatman No. 1 filter paper. The procedure was repeated by resuspending the residue in 200 ml of 0.15 *M* NaCl. All filtrates were combined.

The solubilized proteins were precipitated by the addition of ammonium sulfate until 75% saturation was reached. After 18 hr, proteins were skimmed off the top and dissolved in 40 ml of cold distilled water. The protein solution was dialyzed against distilled water in cellophane tubing for 20 hr. All steps were carried out at 4°C.

Viscometric determinations

The procedure employed in this study was similar to that of Bell, et al. (1955). Ten ml of 1.2% pectin solution, buffered at pH 5 (citrate) were placed in an Oswald viscometer in a water bath maintained at 30°C. Two ml of cranberry dialysates were added to the 10 ml pectin solution making a final concentration of cranberry protein 1%. Flow time readings were made from 0 time to 20 hr, and the percent loss in viscosity was calculated.

Viscosity changes on addition of dialysate were also determined over the pH range 3.0 to 7.0.

Effect of salt and heat on PG activity

The effect of salt was determined at 0.2, 0.4 and 0.6 *M* for 1.2% Sunkist polypectate solution; other conditions were as above.

Test tubes containing the dialysate-substrate solution were heated at 100°C for 5, 10, 15, 25, 35 and 45 min. After heating, the tubes were rapidly cooled and viscosity changes with time were measured in the usual manner.

Cranberry PE

A sample of 0.25 g of acetone powder was dispersed in 10 ml of 0.15 *M* NaCl at pH 7.5 and stirred continuously for 15 min. The dispersion was centrifuged at 25,000 *g* for 15 min and in the supernatant pectin esterase was measured according to the method of Kertesz (1955). The activity was expressed in micro equivalents of ester hydrolyzed per g of acetone powder per hr.

A 1% Sunkist Pectin (3442) was treated with esterase at several pH values. By continuous titration of the assay solution with NaOH, pH values of 5.0, 6.0, 7.0, 7.5 and 8.0 were maintained in different assays. The 1% Sunkist Pectin (3442) was also assayed for esterase activity at pH 7.5 with 0, 0.15 and 0.30 *M* NaCl added.

RESULTS

FIGURE 1 shows the course of action of the three different enzyme extracts on 1%

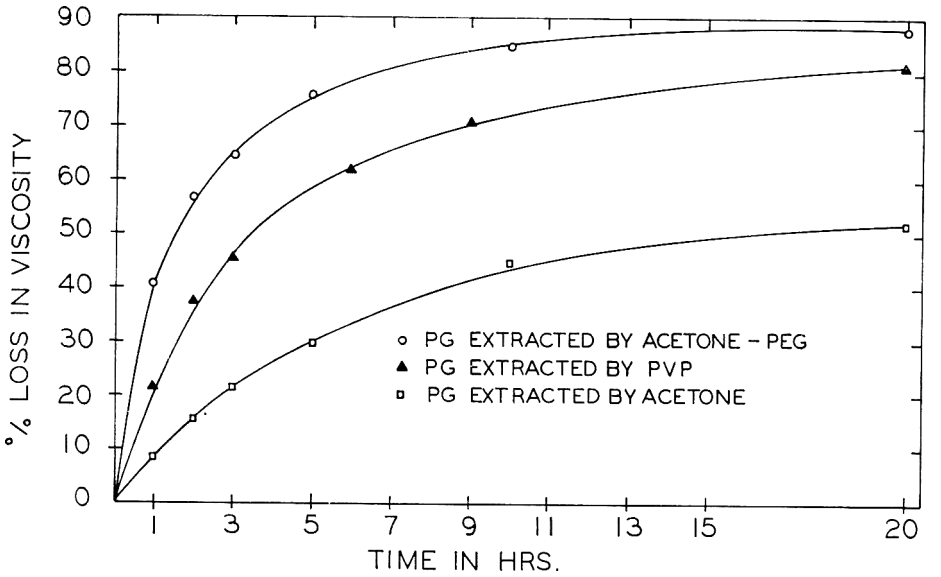

Fig. 1—Percent loss in viscosity of 1% sodium polypectate solution at pH 5.0 and 30°C using cranberry polygalacturonase prepared by three different methods.

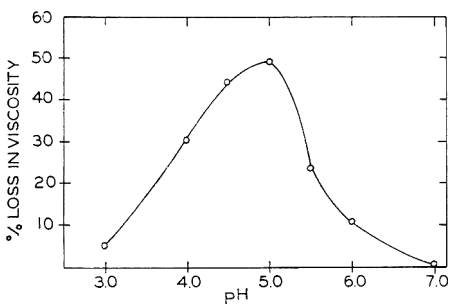

Fig. 2—Effect of pH on cranberry polygalacturonase activity on sodium polypectate at 30°C.

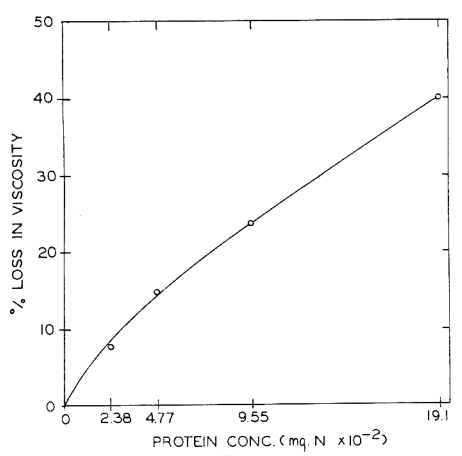

Fig. 3—The relation between different enzyme concentrations of cranberry polygalacturonase and their initial rate of activity on 1% polypectate solution at pH 5.0 and 30°C.

sodium polypectate solution at pH 5.0 and 30°C. After a 40–60% loss in viscosity, the rate of the reaction decreased slowly.

Figure 2 shows the percent loss in viscosity in 1 hr of the solution due to the pectinolytic action of cranberry proteins at several pH values. The highest activity was observed at pH 5.0 and the frequently noted bell-shaped curve was skewed. The influence of sodium chloride at 0.20, 0.40 and 0.60 *M* concentrations on the cranberry pectinolytic activity was very small, as initial rate values corresponding to the change in viscosity during the first hour of the reaction were found to be insignificant.

Figure 3 shows the 1 hr viscosity loss at 4 different protein concentrations. When the rate of activity of the enzyme resulted in a viscosity loss over 15% per hr, a linear relationship existed.

Figure 4 shows the thermal inactivation rate curve of cranberry pectinolytic enzymes. At pH 5.0, the activity of the polygalacturonase was inactivated to about 1% after 35 min of heating at 100°C. McGolloch et al. (1948) indicated that tomatoes contain an unusually heat resistant pectinolytic factor with 20% of the original activity remaining after a heat treatment of 1 hr at 100°C. However, Gizis (1964) found that strawberry pectinolytic enzymes were completely inactivated when treated at 100°C for 35 min.

No transeliminase activity was found in the protein dialysate prepared from the acetone-polyethylene glycol protein preparations, when assayed according to the procedure described by Albersheim et al. (1960).

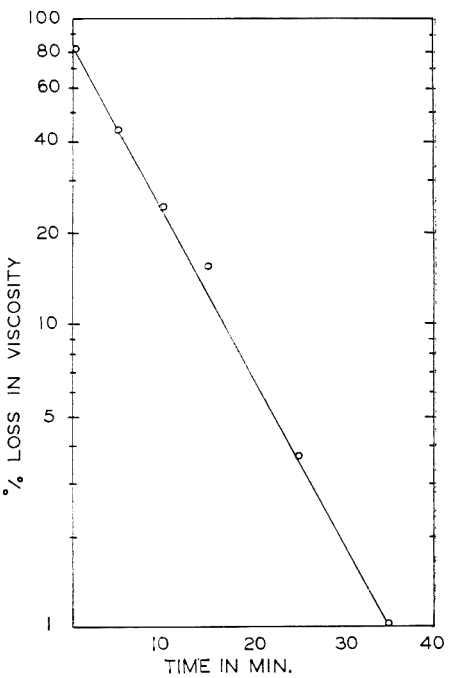

Fig. 4—Thermal inactivation rate curve for the cranberry PG activity on sodium polypectate at pH 5.0 and 30°C.

Figure 5 shows the extent of pectin esterase activity at six different pH values. Figure 6 shows the course of action of cranberry proteins on 1% citrus pectin solution (3442) at pH 7.5 and 30°C. One g of acetone powder was used. According to Kertesz (1955), demethylation of the pectin does not affect the viscosity of their solutions except in the presence of calcium ions. Under such conditions, the gelation properties of the pectin are

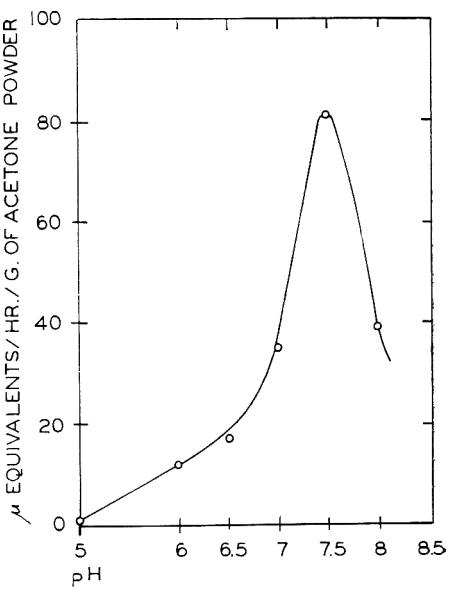

Fig. 5—Activity of PE of cranberries at different pH vales and 30°C, using citrus pectin as substrate.

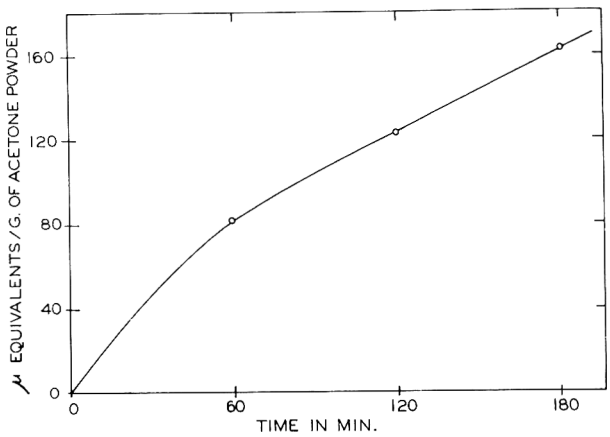

Fig. 6—Cranberry PE activity on 1% citrus pectin solution (0.15M NaCl) at pH 7.5 and 30°C.

Table 1—Effect of NaCl on cranberry pectin esterase activity at pH 7.5.

M NaCl	Activity units of enzyme per g of acetone powder	Activity units/100 g of fresh tissue
0.00	4.0	17.2
0.15	81.0	350.7
0.30	76.0	329.1

Table 2—Effect of heat on cranberry pectin esterase.

Time of exposure to 100°C (min)	μ equivalents/g of acetone powder/hr
0	81.00
1	37.10
2	25.17
3	17.23
5	00.00

affected. Table 1 shows the effect of NaCl on the activity of pectin esterase.

Table 2 shows that cranberry pectin esterase was inactivated when exposed to 100°C for 5 min.

DISCUSSION

THE LOSS in viscosity exhibited by solutions of pectin substances is due to the hydrolysis of the glycosidic linkages of a pectin polymer, thus polygalacturonase is present in cranberries.

The method of enzyme extraction from the cranberry used proves to be essential in obtaining an enzyme extract which exhibits a high rate of activity. Phenol complexing agents prevent the phenol-protein interaction which could cause a decrease in the activity of the enzyme. Figure 1 shows that when the phenol complexing agents are used in extracting the proteins from the cranberries, a higher activity of the polygalacturonase is evident. Moreover, when different types of phenol complexing agents are used, the protein extracts prepared possess different enzyme activity. When the protein was extracted by the use of acetone in conjunction with polyethylene glycol, a higher activity was observed than when the acetone or the polyvinylpyrrolidone protein was used alone. This may be attributed to the stronger affinity of the polyethylene glycol in the presence of acetone to the phenolic compounds.

The activity of the polygalacturonase on the different pectic substances used for substrates was rapid during the initial period of the reaction. This rapid loss in viscosity suggests an interior split of the pectin polymer. Polygalacturonases which possess this type of specificity are referred to as endoenzymes. A slight pectinolytic preference for the citrus sodium polypectate over citrus pectin was evident. The latter possess a higher methoxyl content.

The action of cranberry polygalacturonase was also demonstrated on cranberry water soluble pectin and the calgon soluble pectin. The difference in the activity of polygalacturonase on both was slight. The enzyme showed a considerably higher activity on citrus pectins than on cranberry pectins. This may be attributed to the difference in structure between the pectins of both sources.

The foregoing discussion indicates that the cranberry polygalacturonase possesses the ability to randomly hydrolyze pectin substances with high and low methoxyl content.

Optimum pH for the cranberry polygalacturonase lies in the range of 5.0. The enzyme inactive at neutral pH values is shown in Fig. 2. Although enzyme activity decreases with a decrease in pH, slight activity was observed at pH 3.0. The enzyme was found to be inactivated to within 1% after 35 min at 100°C.

Cranberry proteins exhibited a solubilization effect on the insoluble parent material protopectin. Usually, the concentration of protopectin in fruits decreases with maturation. This may be attributed to its contact with pectic enzymes which may hydrolyze it into the soluble form (Kertesz, 1955).

Pectin esterase activity of the cranberries was low as shown in Figure 6. Optimum pH activity was found at a value of 7.5 with no apparent activity at pH 5.0. Heating enzyme preparations for 5 min at 100°C caused complete inactivation. NaCl at a concentration of 0.15*M* gave an optimum pectin esterase activity. In the absence of the salt, the activity was negligible.

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The Release of Dipicolinic Acid from Spores of Bacillus stearothermophilus NCA 1518

SUMMARY—Both pH of the spore suspensions and autoclaving time affect the release of dipicolinic acid (DPA) from spores. At pH 14 maximum release of DPA was obtained for spore suspensions of both variants autoclaved at 250°F for 15 min. With the smooth variant, maximum release of DPA was achieved at pH 7.0. Autoclaving at 250°F for 15 and 70 min was required for complete release of DPA from spores of the smooth and the rough variants. Loss of viability of spores of both variants succeeded complete release of DPA.

INTRODUCTION

STUDIES of germinating spores of *B. megaterium* led Powell (1953) to isolate and identify dipicolinic acid (DPA). This acid is a strong chelating agent, found in spores in relatively high concentrations—4 to 15% of the dry weight—but could not be detected in vegetative cells (Halvorson et al., 1961).

The acid is released from spores during germination (Powell et al., 1953), or by hydrolyzing the spores (Perry et al., 1955) or by heat (Janssen et al., 1958). The latter is the recommended procedure for DPA analysis.

The two variants—rough and smooth—of this organism differ in their heat resistance. If DPA plays an important role in the thermoresistance of bacterial spores, the rate at which the acid is released should be different for the two variants. It was also of interest to determine the dependence of viability of the release of DPA from the spores.

MATERIALS & METHODS

pH COMBINED with heat and heating time were studied as to their effect on the release of DPA from the spores.

Spore suspensions of the two variants with known "D" values were prepared following the procedures described by Rotman (1967). Spore samples were brought to a concentration of 10^8 spores/ml. The concentration was determined by repeated direct counting, using, a Propper Double Improved Neubauer counting chamber.

Effect of pH

Volumes of 4 ml of samples in 15 × 125 test tubes, were adjusted to pH values of 2.0, 4.0 6.0, 7.0, 8.0, 10.0, 12.0 and 14 with 1.0*N* HCl and 1.0*N* NaOH. These samples were autoclaved for 15 min at 250°F. After cooling the pH values of all samples were adjusted to 6.5, which is the natural pH of exudates of untreated spore suspensions. The sample's volumes were brought up to 5 ml and the concentration of DPA was determined following the procedure described by Janssen et al. (1958).

Effect of Heating Time

Samples of both variants, 5 ml in 15 × 125-mm test tubes, were autoclaved at 250°F for 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80 and 100 min. The concentration of DPA was measured colorimetrically (Janssen et al., 1958).

Viability of the autoclaved spores was determined by ability to: reflect light, absorb malachite green, growth on dextrose tryptone agar and in trypticase soy broth.

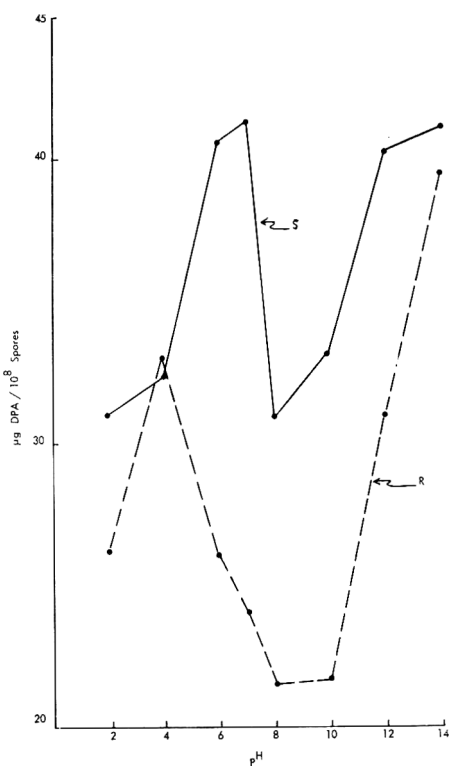

Fig. 1—Effect of pH combined with heat (at 250°F for 15 min.) on the release of DPA from spores of rough and smooth variants.

RESULTS & DISCUSSION

SPORES of the two variants differ in size; those of the rough variant are larger than those of the smooth variant (Rotman et al., 1966). For this reason all analyses were performed on a number of spores basis rather than dry weight basis.

Effect of pH

The release of DPA from spores of both variants is influenced by the pH of the heating menstruum. Spores of the smooth variant had two maxima, one at pH 7.0—41.4 μg DPA/10^8 spores released, and the second at pH 14—41.1 μg DPA/10^8 spores released. The pattern of DPA release from spores of the rough variant exhibited two peaks. The maximum release of DPA occurred at pH 14—39.5 μg DPA/10^8 spores (Fig. 1).

Walker et al. (1965) reported that endospores of aerobic bacilli release the least DPA at pH 7.0, with more acid being released under unfavorable conditions. Similar results were observed in this study. At pH 8.0, both variants released the least amount of DPA. While at this pH value, spores of the smooth variant released 74.4% of the maximum DPA, spores of the rough variant released only 54.5% of the maximum DPA. At pH 6.5 which is the natural pH value of the spore suspensions 63.3 and 98.8% of DPA released from spores of rough and smooth variants, respectively.

To induce complete release of DPA from spores of thermophilic, high heat tolerant organisms, it is recommended to adjust the suspensions to pH 14, prior to autoclaving. To avoid involvement of relatively large volumes and to simplify quantitative analyses, NaOH at concentration of 1.0*N* is desirable.

Effect of Heating Time

The release of DPA from spores is accomplished by autoclaving them at 250°F for 15 min (Janssen et al., 1958). Because of the high heat tolerance of *B. stearothermophilus* spores (Rotman, 1967), it was necessary to determine the effect of autoclaving time on the release

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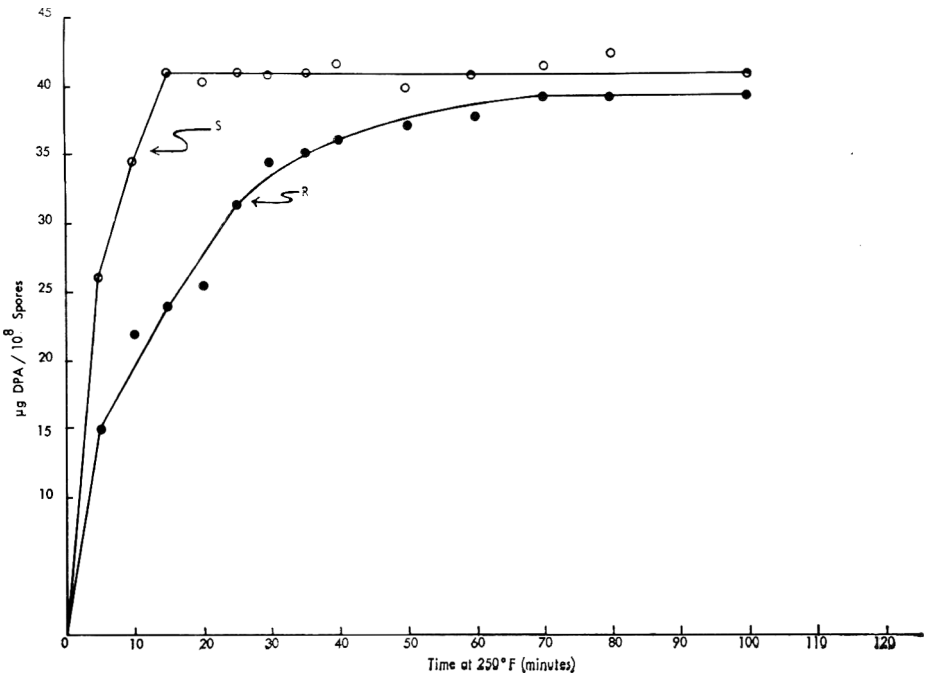

Fig. 2—Effect of autoclaving time at 250°F on the release of DPA from spores of rough and smooth variants (at natural pH of 7.65).

of DPA and viability of the spores.

Maximum release of the acid from spores of the smooth variant was achieved after 15 min of autoclaving at 250°F. For release of DPA, spores of the rough variant had to be autoclaved at 250°F for 70 min (Fig. 2). The maximum levels of DPA obtained were 41.1 and 39.5 µg per 10^8 spores of the smooth and the rough variants, respectively. These results are in complete agreement with the data obtained from the experiment on the effect of pH. Most heat-resistant spores release the least DPA under equal conditions (Walker et al., 1965). Spores of the rough variant were found more heat resistant (Rotman, 1967). When complete release of DPA was achieved, the level of DPA was about equal for both variants. Spores with higher heat resistance, require more adverse conditions to cause complete release of DPA.

The viability of the spores, as related to the release of DPA, was investigated. Spores which had been autoclaved for up to 100 min at 250°F, retained their ability to reflect light and to absorb malachite green, two common characteristics of viable spores. Spores of the smooth variant autoclaved for 30 min and longer did not grow on dextrose tryptone agar at 55°C.

Autoclaving for 50 min inhibited germination of spores of the rough variant on dextrose tryptone agar at 55°C. When autoclaved spores of both variants were inoculated into trypticase soy broth and incubated at 55°C, growth occurred after 72 hr in all tubes including those autoclaved for 100 min.

These results indicate that loss of viability for both variants occurs after the complete release of DPA. Studies with *B. megaterium* spores showed that loss of viability always preceded the complete release of DPA (Rode et al., 1960). Apparently, the role of DPA varies among spores of different species.

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Chemical Composition and Heat Resistance of Bacillus stearothermophilus Spores

SUMMARY—Heat resistance measurement demonstrated that the rough variant was more heat tolerant than the smooth variant. Spores of the two variants were analyzed for DPA, calcium, manganese, magnesium and zinc. No direct relationship was found between DPA, mineral concentrations and heat resistance.

INTRODUCTION

IN A SPECTROCHEMICAL study of different species of bacilli Curran et al. (1943) observed that spores were materially higher in calcium, copper and manganese. This high concentration of calcium was associated with enhanced heat resistance. The calcium content of spores increases during sporulation and it is closely followed by dipicolinic acid (DPA) synthesis (Powell et al., 1956).

High concentration of calcium in the spore might be either a reflection of the relative abundance of calcium in the medium, or enrichment of the medium for spore formation. Slepecky et al. (1959) demonstrated that the spore's metal content is dependent on the concentration and, on the balance of metals in the medium during sporulation.

The purpose was to determine the relationship between the chemical composition and the heat resistance of spores of rough and smooth variants of *B. stearothermophilus* NCA 1518.

MATERIALS & METHODS

SPORE SUSPENSIONS at the concentration of 10^8 spores/ml were prepared following the methods described by Rotman (1967).

Heat resistance studies were performed following the method described by Esty et al. (1924). The procedure is also described in (Rotman et al., 1966).

DPA concentrations were determined by the method of Janssen et al. (1958) after autoclaving the spores for 80 min at 250°F.

Atomic absorption spectrophotometry was used in determination of calcium, manganese, magnesium and zinc. Dry ashing was used in the preparation of the sample. Platinum crucibles containing 20 ml of each spore suspension with 10^8 spores/ml were placed at 500°C overnight. The ash was dissolved in 1 ml of 6*N* distilled HCl. The solution was gently warmed to steaming then transferred into 10 ml volumetric flasks and made to volume with deionized distilled water. Calcium was determined in the presence of 1000 ppm lanthanum (Guillaumin, 1966). Conditions for each analysis were taken from the Analytical Methods book (1964). The Perkin-Elmer Model 290 spectrophotometer was used in all analyses.

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Table 1—Dry weight, ash and concentration of elements in the spores ($\mu g/10^8$ spores).

	Smooth		Rough	
	Concentration	Percent of ash	Concentration	Percent of ash
DPA	41.1	—	39.5	—
Dry weight	181.4	—	138.7	—
Ash	16.5	—	15.2	—
Calcium	6.46	39.2	5.37	35.3
Manganese	0.51	3.1	0.19	1.25
Magnesium	0.29	1.73	9.41	2.66
Zinc	0.07	0.46	0.02	0.12

RESULTS & DISCUSSION

SPORES of the rough variant had higher heat tolerance with a "D" value of 3.5 min, while spores of the smooth variant exhibited a "D" value of 2.33 min.

Results of the chemical analyses are summarized in Table 1.

The two variants did not vary in the concentration of DPA; except for magnesium the smooth variant was richer with the other elements determined. Although the spores of the smooth variant are smaller in size (Rotman et al., 1966), they appear to be heavier. This can attribute to higher concentration of the heavier elements. The spores of this variant were richer with calcium, manganese and zinc.

High concentration of DPA was related to low heat resistance (Byrne et al., 1960). Reports suggested that higher molar ratios Ca:DPA resulted in higher heat tolerance (Levinson et al., 1961) and that calcium was essential to attain maximum thermo-resistance (Foerster et al., 1966). In this study, spores of the smooth variant exhibiting lower heat resistance had higher molar ratios of minerals:DPA (Table 2).

Had the spores been autoclaved for 15 min, as recommended by Janssen et al. (1958), a complete release of DPA would have resulted for the smooth variant, while spores of the rough variant would release only 24 μg DPA/10^8 spores (Rotman, 1967). Using the latter figure in calculating the molar ratios of elements: DPA, the rough variant would have had higher ratio (Table 2). It is doubtful if these molar ratios could serve as valid indicators of heat resistance with this organism.

DPA is present in different chemical combinations in spores of the two variants. This may account for the faster release of DPA from spores of the smooth variant (Rotman, 1967). This rate of DPA release from spores is the key factor in determining the heat tolerance of the corresponding spores.

Table 2—Molar ratios of the elements of DPA.

	Complete release of DPA[1]		Partial release of DPA[2]
	S	R	R
Ca/DPA	0.65	0.57	0.93
Ca + Mn/DPA	0.69	0.58	0.96
Ca + Mn + Mg/DPA	0.74	0.65	1.07
Ca + Mn + Mg + Zn/DPA	0.74	0.65	1.08

[1] Spores of the smooth variant were autoclaved for 15 minutes at 250°F while spores of the rough variant were autoclaved for 80 minutes at 250°F.

[2] Spores of the rough variant were autoclaved for 15 minutes at 250°F.

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A Study on the Sporulation of Rough and Smooth Variants of Bacillus stearothermophilus

SUMMARY—Sporulation of the two variants, rough and smooth, of B. stearothermophilus *NCA 1518 was studied in three complex media, nutrient agar, nutrient broth and trypticase soy agar. The rough variant sporulated best on nutrient agar enriched with one ppm manganese with or without yeast extract but did not sporulate in liquid media. Aerated broth fortified with yeast extract and one ppm manganese was found to be the best sporulating medium for the smooth variant. The effects of calcium, cobalt, and dextrose on the sporulation of the two variants are discussed.*

INTRODUCTION

Bacillus stearothermophilus is the principal flat-sour spoilage organism in low acid, canned foods such as peas, beans and corn. The strain investigated in this study, NCA 1518, exists in two distinct variants, rough and smooth. This classification is based on the appearance of the corresponding colonies on dextrose tryptone agar.

Several theories have been proposed to explain the phenomenon of sporogenesis. Lack of nutrients in the sporulation medium was believed a major factor in inducing spore formation. Fabian et al. (1933) showed that sporulation occurred at the point of maximum cell viability. However, Knaysi (1945) reported that "endospores are formed by healthy cells facing starvation." Later, Grelet (1952) observed that sporulation is the genetic response of the cell to an increase in the generation time. The conditions which favor sporulation reduce or stop the cell's growth.

Many factors affect sporulation. Manganese initiates and increases the rate of sporulation (Charney et al., 1951). On the other hand, compounds such as glutamate salts act as inhibitors or as abating agents of the sporulation process (Foster et al., 1949). Little is known about the differences in sporulation requirements between variants of the same species. This study attempts to determine some of these requirements for each variant, and how they might be different.

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MATERIALS & METHODS

PURE CULTURES of rough and smooth variants of *B. stearothermophilus* NCA 1518, isolated by the single cell technique (Rotman et al., 1966) were used in this investigation. Cells of both variants, thinly coated on glass beads, were kept frozen to serve as starter inocula. Dextrose tryptone agar plates were inoculated by using a single bead. The plates were incubated for 24 hrs at 55°C to serve as fresh active inocula for spore production studies.

Three complex media were used in this investigation: nutrient agar and nutrient broth (obtained from Difco Laboratories, Detroit, Mich.); and trypticase soy agar, hereafter TSA (obtained from B. B. L., Baltimore, Md.). The effect of added yeast extract, 0.4% (obtained from Fisher Scientific Company), dextrose (0.05%), and 1–10 ppm of calcium (as $Ca(NO_3)_2 \cdot 4H_2O$), cobalt (as $Co(NO_3)_2 \cdot 6H_2O$), and manganese (as $MnSO_4 \cdot H_2O$) on stimulation of sporulation was studied.

Sporulation was carried over a short period of 48 hr at 55°C to avoid non-homogeneous population as a result of germination (Rotman, 1965, unpublished data). Standard petri-dishes were used for sporulation studies on solid media. For sporulation in broth, volumes of 200 ml of liquid media were placed in one-liter Erlenmeyer flasks with a magnetic stirring bar ($2'' \times 3/8''$) in each. Continuous stirring provided aeration. The stirring speed was adjusted to give the deepest possible vortex without foam formation.

All glassware used in this research was autoclaved 5–6 hr to insure complete mineral leaching out of the glass. Deionized water was used in the preparation of the media and the supplement solutions.

The degree of sporulation was determined by direct microscopic counts. Duplicate plates were prepared for each treatment. The degree of sporulation was determined by direct microscopic counts of two slides prepared from each plate. Five randomly selected fields were examined in each slide.

RESULTS & DISCUSSION

THE DEGREE of sporulation of both variants on solid media largely depends on the size of the inoculum. Heavy inoculum was required for the rough variant, whereas better sporulation of the smooth variant occurred with a light inoculum.

Effect of calcium on the sporulation on nutrient agar

Sporulation of the smooth variant was not affected significantly by adding calcium to the medium (Fig. 1). Calcium in the medium, however, caused an increase in the percentage of "red" spores formed. (Red spores is a term used for immature spores which exhibit a red periphery after staining with malachite green and safranine. Mature spores stain green uniformly.)

At the levels tested, calcium had a partial inhibitory effect on the sporulation of the rough variant (Fig. 1). Unlike the smooth variant, no "red" spores were observed with the rough variant after 48 hr of incubation.

Incorporating dextrose resulted in complete inhibition of sporulation for both variants at all calcium concentrations tested. Dextrose enhances the partial inhibition effect exerted by the calcium.

Effect of cobalt on the sporulation on nutrient agar

This element was studied because of its biological importance. Cobalt belongs to the first transition group of elements and is notable for its toxicity and ability to form complexes. Since this element is important in the activation of certain oxidative enzymes, it was hypothesized that its presence in the sporulation medium might increase the rate of sporulation.

Cobalt's effect on the sporulation of the two variants is shown in Figure 2. These results indicate that cobalt in the medium tends to reduce sporulation of the smooth variant. At 6 ppm and higher concentrations, cobalt moderately stimulated sporulation of the rough variant after 24 hr of incubation. Further incubation initiated germination of previously formed spores and prevented formation of new spores. Cobalt chelates compounds essential for sporulation, thus altering the normal process of sporulation.

Dextrose, when added to the medium containing cobalt, completely inhibited spore formation of both variants.

Effect of manganese on sporulation on nutrient agar

The smooth variant sporulated poorly on nutrient agar supplemented with manganese (Fig. 3), with most of the spores exhibiting red peripheries. After 24 hr of incubation, there was some decrease in spore formation as the concentration of manganese increased. Although the significance of this phenomenon is unclear, it seems to support the information reported by Levinson et al. (1955), who observed that manganese stimulates respiration and consequently germination of spores.

Addition of manganese to nutrient agar distinctly affected the degree of sporulation of the rough variant. The addition of 1 ppm manganese resulted in the highest spore yield (86%, Fig. 3). Although after 24 hr of incubation the major portion of the spore crops consisted of "red" spores, an additional 24 hr of incubation resulted in a decrease in the abundance of "red" spores to a negligible level. Higher levels of maganese lowered the degree of sporulation, due to partial germination, stimulated by high concentrations of the element.

When dextrose was incorporated into the medium, sporulation of the smooth variant was completely inhibited for all

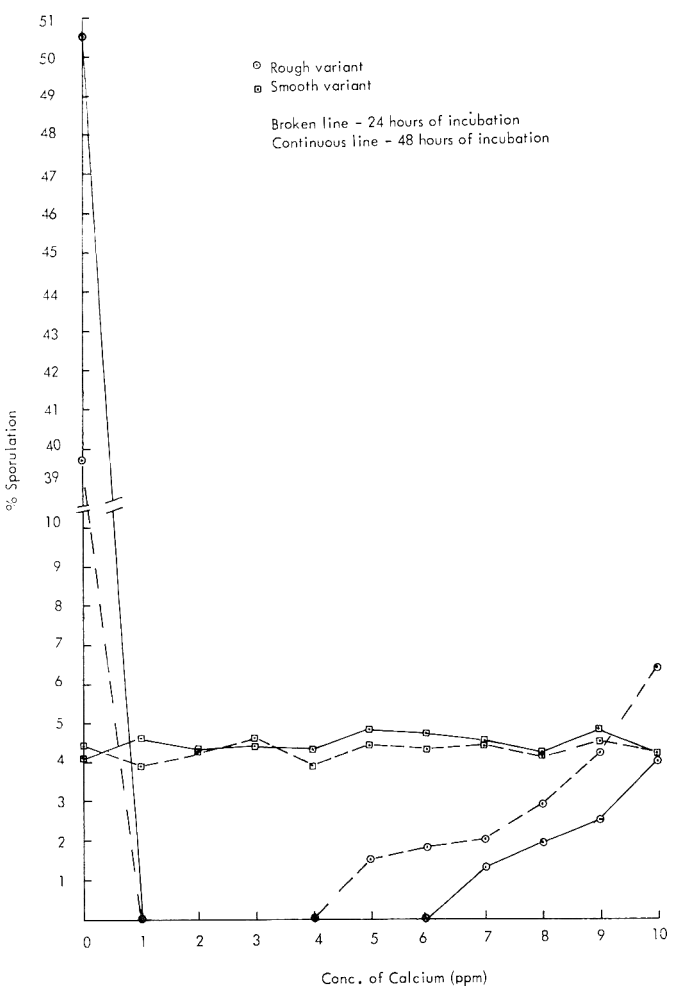

Fig. 1—Effect of calcium on sporulation on nutrient agar.

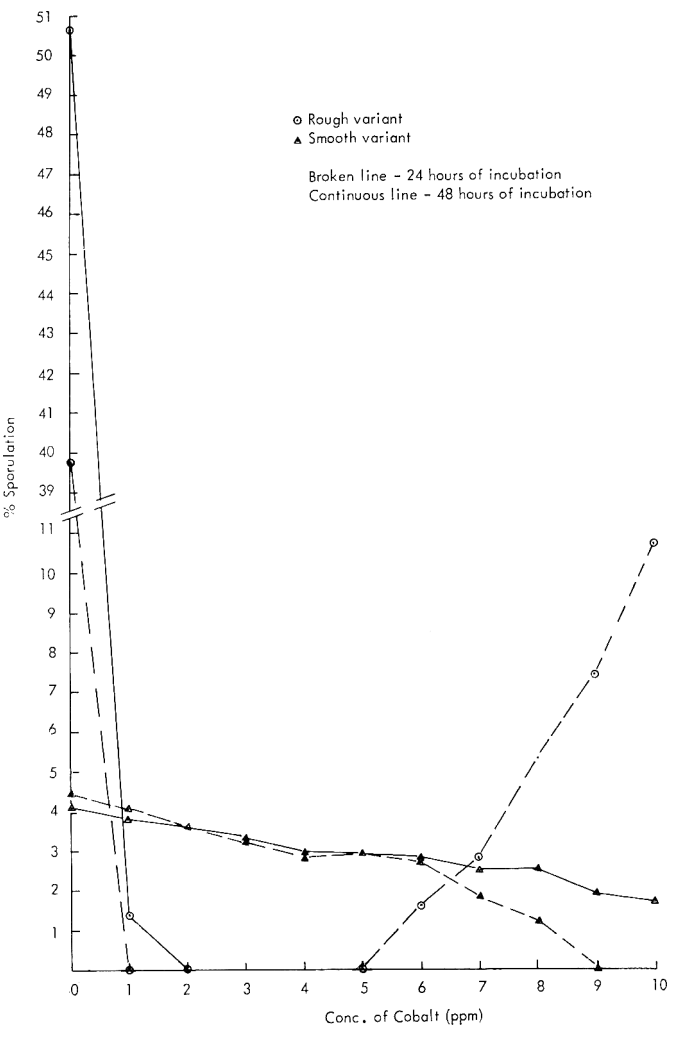

Fig. 2—Effect of cobalt on sporulation on nutrient agar.

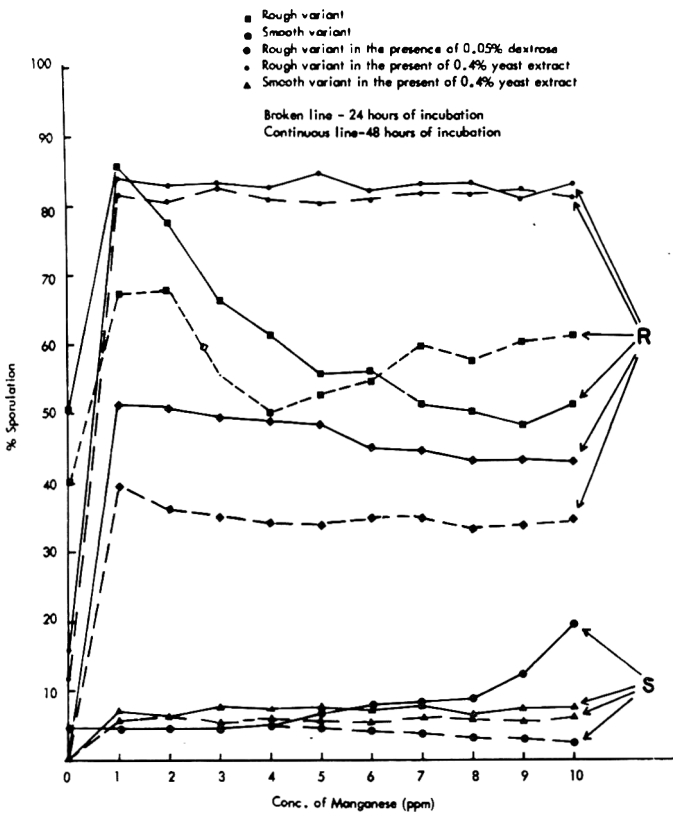

Fig. 3—Effect of manganese on sporulation on nutrient agar.

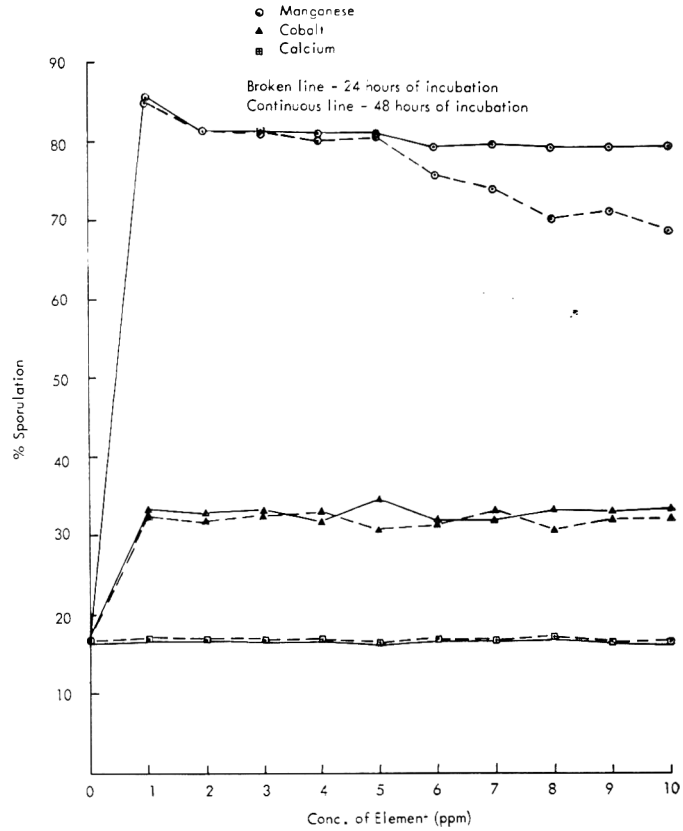

Fig. 4—Sporulation of the smooth variant in aerated nutrient broth fortified with 0.4% yeast extract.

levels of manganese tested after both 24 and 48 hr of incubation. Similar results were observed for the rough variant when no manganese was added.

Halvorson (1957) suggested that dextrose interferes with some enzymic steps essential for the sporulation process. Addition of 1 ppm managanese counteracted the inhibitory effect of the dextrose on the sporulation of the rough variant (Fig. 3). Similar sporulation was obtained for this variant in the absence of glucose and manganese. These observations offer convincing evidence that the sporulation mechanisms are different for the two variants.

Effect of yeast extract on sporulation on nutrient agar

The effect of yeast extract was studied in combination with manganese only, because of the inhibitory effects by calcium and cobalt on the sporulation of the two variants. In the absence of manganese, no spores were observed with the smooth variant. Adding manganese to the medium caused only a moderate increase in spore formation by this variant (Fig. 3), with most of the spores being mature.

Sporulation of the rough variant on nutrient agar, enriched only with yeast extract, was relatively low. Incorporation of manganese caused a significant increase in sporulation (Fig. 3). Although higher concentrations of manganese did not increase the sporulation, many "red" spores were observed at higher levels of the element after 24 hr of incubation. Additional 24 hr of incubation resulted in homogeneous, mature spore crops at 1–7 ppm manganese and negligible levels of "red" spores at 8–10 ppm of the element.

It is evident that yeast extract st mulated sporulation of the rough variant to a higher degree than that of the smooth variant.

Sporulation on trypticase soy agar (TSA)

Sporulation of both variants was inhibited by low levels of dextrose. TSA was examined as a possible medium of sporulation. No spores were formed by either variant on this medium in the absence and presence of manganese, calcium and cobalt.

Sporulation in nutrient broth

Sporulation of both variants did not occur in still and aerated plain broth. Adding dextrose at inoculation time did not stimulate spore production. Attempts to stimulate spore production by addition of manganese, calcium and cobalt were unsuccessful.

Incorporating yeast extract into the medium stimulated sporulation of the smooth variant in the aerated culture, but not in a still culture. Fortification of the aerated broth with manganese resulted in increased sporulation to a maximum level (85%) at the concentration of one ppm manganese (Fig. 4). Under these conditions no "red" spores were formed at any manganese levels tested.

While calcium did not stimulate higher sporulation of the smooth variant in aerated nutrient broth fortified with yeast extract, cobalt did (Fig. 4). At the levels tested cobalt doubled the degree of sporulation. With higher cobalt concentrations, more "red" spores were formed after 48 hr of incubation. Calcium and cobalt did not stimulate sporulation of the smooth variant in still culture of yeast extract nutrient broth.

The rough variant did not sporulate in still and aerated cultures under the various treatments. This observation is of great significance, since by this method one can prepare a spore suspension of the smooth variant with high degree of purity. If rough cells are present in the original inoculum, they will outgrow the smooth variant which, in turn, will not produce spores. These results are in accord with those found by Hill (1967), who reported a shorter generation time for the rough variant in nutrient broth.

The rough variant's inability to sporulate in variously treated liquid medium supports the concept of different sporulation mechanisms and requirements for the two variants. The rough variant requires

very low oxygen tension for sporulation while the smooth variant requires an ample supply of oxygen to sporulate. Both variants require manganese to stimulate sporulation. The smooth variant did not sporulate in broth in the absence of yeast extract; the rough variant sporulated well on nutrient agar with or without yeast extract. Further investigation is required to determine the effect of vitamins, amino acids and other yeast extract components on the sporulation of the two variants.

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Colorimetry of Foods. 1. Correlation of Raw, Transformed and Reduced Data with Visual Rankings for Spinach Puree

SUMMARY—Freshly processed spinach purée and stored processed purée were mixed in proportions varying from 0 to 100% in increments of 10% to provide three different sets of 11 samples each. These sets simulated the range of color values actually obtained with samples in storage after processing. Color measurements were performed by means of a General Electric Recording Spectrophotometer, a Hunterlab Model D25 Color Difference Meter, and a Colormaster Differential Colorimeter, Model V. Experiments were carried out on each set of samples to obtain tristimulus values from each instrument. These values were mathematically transformed to the tristimulus values of the other two color systems. Tristimulus values from the instruments also were reduced to common color functions, and all data were correlated with visual rankings. Good correlations of instrument versus visual rank were obtained. Absolute values obtained from the transformation equations were very different from instrumental values. However, the transformations resulted in only a slight decrease in correlation with visual preference. Reduced data calculated from instrumental read-out correlated well with visual rank. However, conversion of Colormaster data to Adams data and resultant reduction produced poor correlations.

INTRODUCTION

A GREAT DEAL of effort has been aimed at stabilizing the green pigments of thermally processed vegetables. Many different methods have been attempted with varying degrees of success. Elevated pH conditions created in the food by the addition of improved alkaline substances have seen a great deal of popularity (Blair, 1940a,b; Stevenson et al., 1942; Benedix et al., 1952; Gupte et al., 1964; and Malecki, 1965).

Another approach has been the conversion of chlorophylls to chlorophyllides with or without the addition of $MgCO_3$ as summarized by Clydesdale et al. (1968a). Still another has been the use of High-Temperature Short-Time processing as evidenced by the work of Epstein (1959), Tan et al. (1962), Gupte et al. (1964), and Luh et al (1964).

The evaluation of the results in most of these cases has emphasized pigment content as a measure of success. This criterion is an extremely important one but, in the final analysis, visual acceptance of the product by a consumer will ultimately predict the success of a process. This leads to the need for an adequate means of properly assessing the color of a food material.

Instrumental color measuring techniques are becoming much more popular in food research as a means of obtaining a valid objective measurement of color. Moreover, the tristimulus values obtained from an instrument may be reduced to obtain one value that describes the color or converted to some other color system to make the definition of the color simpler. This practice in theory is quite legitimate and at times very successful. Unfortunately there are cases, as Clydesdale et al. (1968a) have pointed out, where correlations do not exist between converted and/or reduced data and visual judgments.

This research was carried out to investigate the validity of using converted and/or reduced data in formulating a prediction of visual judgment on processed spinach purées.

The color conversions are known to be valid with opaque color standards and the purpose of this work was to check their validity with a translucent food sample such as spinach purée.

EXPERIMENTAL

Preparation of sample

Fresh spinach obtained from a local supermarket was washed, blanched at 185°F for 3 min and drained. The leaves were comminuted in a Fitzpatrick Mill (W.J. Fitzpatrick Co., Chicago). Following comminution, the purée was deaerated using a vacuum desiccator connected to a water aspirator. The deaerated purée was then filled into baby food jars (201x210) and sealed by means of a steam vacuum sealer (White Cap Co., Chicago) with a pressure of 5 lb.

The filled jars were processed according to the heat penetration data recommended by Tan et al. (1962) to give an Fo = 4.9.

Freshly processed purées and stored processed purées were mixed in proportions varying from 0 to 100% in increments of 10% to provide a series of 11 samples. These

samples simulated the range of color values actually obtained with samples in storage after processing. With each set of experiments, a different stored processed purée was chosen to vary the range of colors slightly.

Table 1—Correlations of visual rankings versus instrumental tristimulus values for processed spinach purées.

Correlation. Visual ranking with:		Correlation coefficient I	II	III[1]
			(r)	
Colormaster	G	0.959	0.980	0.986
	R	0.956	0.979	0.987
	B	0.879	0.968	0.979
Hunter	L	0.961	0.959	0.992
	a	0.945	0.993	0.976
	b	0.979	0.830	0.994
G.E.	X	0.960	0.979	0.990
	Y	0.968	0.980	0.989
	Z	0.948	0.876	0.909
			(R)	
Colormaster	G,R	0.960	0.981	0.989
	G,R,B	0.975	0.984	0.989
Hunter	a,b	0.986	0.993	0.995
	L,a,b	0.986	0.993	0.996
G.E.	X,Y	0.976	0.983	0.990
	X,Y,Z	0.976	0.989	0.997

[1] I, II and III refer to three separate experiments, each having 11 samples.

A total of three different sets of samples were prepared and examined in three different experiments.

Subjective evaluation of color

Visual judgments for each set of samples were performed with 25 panel members. All panel members were instructed to rank the samples from 1 to 11 with the rank of 1 being assigned to the sample with the most acceptable color for a commercial spinach purée and 11 assigned to the least acceptable. Controlled illumination of the samples was obtained by the use of a MacBeth Examolite (MacBeth Daylighting Corp., Newburgh, N.Y.) with horizon sunlight. Each set of samples was ranked in duplicate and the results averaged.

Objective evaluation of color

Instrumental color data were obtained with three instruments: a Hunterlab Model D25 Color Difference Meter (Hunter Associates Laboratory, Inc., Fairfax, Va.), a Colormaster Differential Colorimeter, Model V (Manufacturers Engineer and Equipment Co., Hatboro, Pa.), and a General Electric Recording Spectrophotometer.

The choice of these instruments should not be interpreted as an endorsement. They were chosen to obtain data in three different tristimulus systems. These values could then be interconverted mathematically to investigate the validity of the use of transformation equations with the food samples under examination.

The standards used with each instrument were as follows.

Hunterlab D25: Gray tile with tristimulus values L = 23.8, a = 1.9, b = −0.7

Colormaster Model V: Gray tile with tristimulus values G = 5.52, R = 4.49, B = 6.22

G.E. Recording Spectrophotometer: a pressed barium sulfate standard.

Duplicate measurements were made on each set of samples on the same day as the visual examinations. The average values of the duplicates were used in the analysis of the data. All samples were measured using a depth of 2 cm in each cell.

All instruments were used according to the directions outlined by the manufacturer.

Interconversion of color data

Each set of tristimulus values obtained instrumentally was converted by the standard conversion equations to the other tristimulus values by means of the computer program developed by Clydesdale et al. (1968b). The Colormaster GRB data were converted to Adams L,a,b data by the format supplied by the manufacturer.

Analysis of data

The analysis of data may most conveniently be described by division into four sections:

1. Instrumental tristimulus values were correlated to visual ranking to investigate the function or functions best correlated with visual preference.
2. Color values from each instrument were tabulated together with those calculated in the same system but obtained from the other two instruments. All values were then correlated with visual rankings to determine the validity of the use of transformation equations in defining the color of processed spinach purée.
3. Instrumental and calculated tristimulus values were reduced to such color functions as a/b, $\tan^{-1}$ a/b, $(a^2 + b^2)^{1/2}$ G/R, and X/Y. In the case of G,R,B, a conversion to Adams L,a,b tristimulus values was carried out (there is no instrumental method for obtaining Adams L,a,b and therefore these values were considered as reduced data). The Adams data were further reduced to a/b, $\tan^{-1}$ a/b, and $(a^2 + b^2)^{1/2}$. All reduced data were correlated against visual ranking to investigate their relationship to the subjective evaluations.
4. ΔE values, a measure of total color difference were calculated between the most and least acceptable samples in each experiment. The series of samples used in each experiment were purposefully created to have not only different total color differences but also to have color differences that verged on the visual threshold for color discrimination. These factors could be tested only by means of an objective color difference score. Therefore ΔE values were calculated to ensure that the experimental objectives had been met. Large visual differences are much easier to measure objectively, and this work was designed to investigate problems associated with small visual differences.

Table 2—Visual color rankings versus instrumental G.E. and calculated CIE X, Y, Z data obtained for processed spinach purées.

Visual rank	Tristimulus values: Read-out from G.E. X	Y	Z	Calculated from Hunter D25 data X	Y	Z	Calculated from Colormaster data X	Y	Z
1.26	9.31	9.71	6.44	5.17	5.43	1.58	5.81	5.88	1.43
2.34	9.05	9.41	6.38	4.87	5.11	1.49	5.43	5.49	1.36
3.16	8.82	9.17	6.33	4.63	4.84	1.41	5.17	5.22	1.30
3.63	8.60	8.92	6.28	4.44	4.62	1.40	4.94	5.00	1.25
5.00	8.49	8.79	6.31	4.63	4.80	1.42	4.67	4.71	1.17
6.00	8.27	8.55	6.20	4.51	4.67	1.43	4.53	4.57	1.16
7.37	8.18	8.44	6.19	4.36	4.49	1.37	4.37	4.42	1.13
8.32	8.09	8.35	6.25	4.21	4.33	0.97	4.21	4.26	1.09
8.75	7.96	8.19	6.16	4.10	4.20	1.30	4.07	4.09	1.04
9.80	7.91	8.13	6.23	4.03	4.12	1.30	4.03	4.05	1.06
10.40	7.77	7.99	6.17	3.88	3.96	1.29	3.89	3.89	1.04

Table 3—Correlations of visual rankings versus C.I.E. X, Y, Z data obtained with a General Electric Recording Spectrophotometer and also calculated from other systems.

Correlation. Visual ranking with:	Correlation coefficient 1 I	II	III	2 I	II	III	3[1] I	II	III
					(r)				
CIE X	0.960	0.979	0.990	0.939	0.947	0.991	0.944	0.979	0.987
CIE Y	0.968	0.980	0.989	0.957	0.956	0.989	0.959	0.980	0.987
CIE Z	0.948	0.876	0.909	0.848	0.695	0.976	0.883	0.968	0.976
					(R)				
CIE X,Y,Z	0.976	0.989	0.997	0.987	0.988	0.996	0.984	0.984	0.989

[1] 1—CIE X,Y,Z read-out from G.E. Recording Spectrophotometer; 2—CIE X,Y,Z calculated from Hunter D25 L,a,b; 3—CIE X,Y,Z calculated from Colormaster Model V G,R,B.

[2] I, II and III refer to the average of duplicates of each of three separate experiments.

RESULTS & DISCUSSION

Correlation with actual data

Table 1 shows the correlations obtained between instrumental tristimulus values and visual ranking for each of three separate experiments. As expected, data from all the instruments produced high correlations in all three experiments. The third tristimulus value (Colormaster B, Hunter b, and G.E. Z) were lower in at least one of the experiments. This was probably because this tristimulus value is a measure of the energy of the visible spectrum concerned with lower wavelengths, i.e., blue. Apparently blue is less important in color judgments of processed spinach purée than the other two tristimulus values.

The multiple correlations (Table 1) are higher than the simple correlations but the improvement was not marked. This was because, except in the case of B, b or Z, the simple correlations were already very high.

Correlations with calculated data

Tables 2, 4 and 6 show the instrumental and calculated tristimulus values obtained from the G.E. Recording Spectrophotometer, the Hunterlab D25, and the Colormaster Model V, respectively, for Expt. II. Tables 3, 5 and 7 show the results of correlation of these data with visual ranking.

The transformation to X,Y,Z from other systems (Table 2) resulted in very different absolute values than that obtained when X,Y,Z were obtained directly from the G.E. Recording Spectrophotometer. This indicates that the spinach purée, a translucent sample, does not behave like an opaque standard on which the transformation equations are based. This observation is what might be expected when equations developed for one system are applied to a completely different system and one that contains far from ideal opaque samples.

However, although the absolute values were changed drastically, the values in relation to ranking of color were not affected to the same degree as evidenced by the correlations presented in Table 3. This was the case with all three experiments but the correlations with calculated values were not, in general, as good as the values obtained from the instrument. This shows one of the dangers implicit in using transformations on a food material when visual judgments are not available.

Tables 4 and 5 present the transformations and correlations, respectively, of results obtained for the Hunter L,a,b color solid. Table 4 shows the instrumental and calculated values of L,a,b obtained from Experiment II. Again the absolute values were different from instrumental values but were not changed to the same extent as the X,Y,Z values. However, the correlations in Table 5 show that in Experiments I and II when Hunter a was calculated from G,R,B data the correlations with visual ranking were decreased to an appreciable extent. Hunter a is a common function used to describe green vegetable color since it is a measure of greenness (−a, green; +a, red) and obviously in this transformation the function has lost its value in measuring visual judgments.

Tables 6 and 7 show the same type of results for Colormaster G,R,B data, and the precautions for using transformation equations previously cited also apply in this case.

The correlations changed slightly with each set of experiments, perhaps because the sample series varied slightly in hue,

Table 4—Visual color rankings versus Hunterlab D25 and calculated Hunter L,a,b data obtained for processed spinach purées.

Visual rank	Tristimulus values: Read-out from Hunter D25 L	a	b	Calculated from G.E. data L	a	b	Calculated from Colormaster data L	a	b
1.26	23.3	−1.2	12.3	31.16	−1.20	9.56	24.25	0.30	13.48
2.34	22.6	−1.1	11.9	30.68	−1.02	9.14	23.43	0.39	12.97
3.16	22.0	−0.9	11.6	30.28	−1.00	8.80	22.85	0.37	12.62
3.63	21.5	−0.8	11.2	29.87	−0.87	8.44	22.36	0.29	12.33
5.00	21.9	−0.6	11.5	29.65	−0.77	8.14	21.70	0.40	12.00
6.00	21.6	−0.5	11.2	29.24	−0.69	7.90	21.38	0.42	11.75
7.37	21.2	−0.4	11.0	29.05	−0.58	7.70	21.02	0.31	11.52
8.32	20.8	−0.3	11.8	28.90	−0.60	7.40	20.64	0.25	11.33
8.75	20.5	−0.2	10.6	28.62	−0.43	7.27	20.22	0.56	11.12
9.80	20.3	−0.1	10.4	28.51	−0.38	7.01	20.13	0.52	10.96
10.40	19.9	0.0	10.1	28.27	−0.40	6.85	19.72	0.71	10.68

Table 5—Correlations of visual rankings versus Hunter L,a,b obtained from a Hunter D25 and also calculated from other systems.

Correlation, Visual ranking with:	Correlation coefficient: 1 I	1 II	1 III	2 I	2 II	2 III	3[1] I	3[1] II	3[1] III
					(r)				
Hunter L	0.961	0.959	0.992	0.964	0.985	0.990	0.959	0.982	0.996
Hunter a	0.945	0.993	0.976	0.741	0.611	0.972	0.924	0.983	0.997
Hunter b	0.979	0.830	0.994	0.972	0.987	0.991	0.963	0.989	0.999
					(R)				
Hunter L,a,b	0.986	0.993	0.996	0.986	0.988	0.991	0.979	0.993	0.998

[1]1—Hunter L,a,b read-out from Hunter D25; 2—Hunter L,a,b calculated from Colormaster Model V G,R,B; 3—Hunter L,a,b calculated from G.E. Recording Spectrophotometer X,Y,Z.
[2] I, II and III refer to the average of duplicates of each of three separate experiments.

Table 6—Visual color rankings versus Colormaster and calculated G,R,B data obtained for processed spinach purées.

Visual rank	Tristimulus values: Read-out from Colormaster G	R	B	Calculated from Hunter D25 data G	R	B	Calculated from G.E. data G	R	B
1.26	5.88	7.11	1.21	5.43	6.26	1.33	9.71	10.52	5.45
2.34	5.49	6.65	1.15	5.11	5.90	1.27	9.41	10.20	5.40
3.16	5.22	6.32	1.10	4.84	5.62	1.19	9.17	9.92	5.36
3.63	5.00	6.04	1.06	4.62	5.37	1.18	8.92	9.65	5.32
5.00	4.71	5.71	0.99	4.80	5.61	1.20	8.79	9.50	5.34
6.00	4.57	5.54	0.98	4.67	5.46	1.21	8.55	9.24	5.25
7.37	4.42	5.34	0.96	4.49	5.27	1.16	8.44	9.13	5.24
8.32	4.26	5.14	0.92	4.33	5.17	0.82	8.35	9.00	5.29
8.75	4.09	4.98	0.88	4.20	4.96	1.10	8.19	8.85	5.22
9.80	4.05	4.92	0.90	4.12	4.87	1.11	8.13	8.78	5.28
10.40	3.89	4.75	0.88	3.96	4.68	1.09	7.99	8.61	5.22

Table 7—Correlations of visual rankings versus Colormaster G,R,B obtained from a Colormaster Model V and also calculated from other systems.

Correlation. Visual ranking with:	Correlation coefficient								
	1			2			3[1]		
	I	II	III	I	II	III	I	II	III[2]
					(r)				
Colormaster G	0.960	0.980	0.986	0.957	0.956	0.989	0.956	0.980	0.987
Colormaster R	0.956	0.980	0.987	0.941	0.945	0.992	0.939	0.980	0.991
Colormaster B	0.879	0.968	0.979	0.848	0.686	0.975	0.933	0.878	0.907
					(R)				
Colormaster G,R,B	0.975	0.984	0.989	0.986	0.987	0.996	0.980	0.988	0.998

[1] 1—Colormaster G,R,B read-out from Colormaster Model V; 2—Colormaster G,R,B calculated from Hunter D25 L,a,b; 3—Colormaster G,R,B calculated from G.E. Recording Spectrometer X,Y,Z.
[2] I, II and III refer to the average of duplicates of each of three separate experiments.

Table 8—Correlations of visual rankings versus reduced data for processed spinach purées.

Correlation. Visual ranking with:	Correlation coefficient		
	I	II	III
		(r)	
Colormaster G/R	0.809	0.744	0.937
Adams L (from Colormaster data)	0.965	0.986	0.925
" a " " "	0.769	0.491	0.981
" b " " "	0.968	0.968	0.993
" a/b " " "	0.772	0.584	0.991
" $\tan^{-1}$ a/b " "	0.772	0.580	0.991
" $(a^2 + b^2)^{1/2}$ " "	0.969	0.969	0.992
Hunter a/b	0.918	0.995	0.989
" $\tan^{-1}$ a/b	0.919	0.995	0.989
" $(a^2 + b^2)^{1/2}$	0.976	0.840	0.979
G.E. X/Y	0.841	0.857	0.964

value and chroma as well as in total color differences between the first and last sample. The samples were carefully chosen to ensure small color differences in these parameters.

Correlations with reduced data

In many cases the incorporation of two tristimulus values into one function will provide a single measure of color that simulates visual judgment. This has been termed "reduction of data" and the results of such treatment are shown in Table 8.

In the case of G/R and X/Y the result was to produce correlations with visual ranking, which were not as good as the single tristimulus values alone. Obviously there is no advantage to using this type of reduction for the samples used in this study.

Single Adams' tristimulus values are also presented. These values do not strictly fall under the definition of "reduced data" but they are included in this table since there is no instrumental method to measure Adams' L, a, and b. Only Adams' b and L showed a good correlation with visual judgment. It was known from the other measurements in this study that L (lightness or darkness) was a good measure of visual preference for the samples under study; however, b was not as good. This indicates that the Adams transformation caused b to correlate more directly with visual preference than it actually should.

Adams' a value showed a significantly poorer correlation in two of the experiments than any other functions associated with an a value. Thus the transformation has lowered the correlation of a, which is normally quite a good indication of visual preference for green food samples.

Adams a/b and $\tan^{-1}$ a/b also showed low correlations with visual judgment. The Adams functions are valuable tools when used properly (i.e., with opaque materials). However, they do not seem to produce valid results with the samples used in this study. Since they have been used widely it should be stressed that each individual type of colored material should first be ranked visually to establish good correlation with Adams data before transformations are used to predict visual preference.

Adams $(a^2 + b^2)^{1/2}$ which is a function of chroma, showed good correlations with visual rank and apparently has solved some of the problems associated with the a value.

The Adams a, a/b, and $\tan^{-1}$ a/b correlated well with visual judgments (Table 8) in only one of three experiments. The series that showed the poorest correlation (Expt. II) had a total color difference between the first and last samples of 4.2 as measured with the D25 and calculated by

$$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$$

The series with the intermediate correlation (Expt. II) had a ΔE of 6.0, whereas the one that correlated most highly had a ΔE of 6.2. Apparently, with spinach purée, as the total color difference decreases, the validity of the transformation decreases.

As was expected, since Hunter a and b values were high in correlation with visual ranking, the reduced data showed the same type of correlation with the exception of $(a^2 + b^2)^{1/2}$, which was slightly lower in one of the three experiments.

In general, this study showed the value of instrumental color measurement, as all instruments performed well when the raw instrument data were used. If it is desired to convert the color data to another system or to reduce the number of parameters, more care in interpretation is required.

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Determining Glucose and Glycogen from a Single Sample of Meat

SUMMARY—A method was developed determining glucose and glycogen as separate entities from a single sample of meat. This method is based on the quantitative separation of glycogen from glucose by precipitation of the former species with an inert organic solvent. During this separation care must be exercised not to allow the temperature of the meat extract to rise above 5°C. After separation the two carbohydrate species may be analyzed by existing methods. The accuracy of this method was tested by comparing its results with those of established methods for the determination of glucose and glycogen. The error caused by the formation of Maillard addition products was evaluated.

INTRODUCTION

THE METHODS used for the determination of glucose and glycogen in meat require two separate meat samples, one for glycogen and one for glucose. This paper presents a method for the determination of both carbohydrate species from a single sample.

A method for the analysis of glucose in meat was developed by Somogyi (1952). This method uses water to extract the glucose from a finely ground meat sample. The interfering proteins in the aqueous extract are precipitated with heavy metal salts. The resultant deproteinized glucose solution is used to reduce a copper-tartrate reagent, which is reacted with an arsenomolybdate solution to form a highly colored complex. The absorbance of this complex is directly proportional to the amount of glucose present in the sample.

A method used for the analysis of glycogen in meat was developed by Good et al. (1933). This method uses an aqueous KOH solution to extract glycogen from a meat sample. The glycogen is precipitated from the alkaline extract with ethanol and is then hydrolyzed to glucose and determined by copper reduction.

These two methods cannot be employed together on a single meat sample for two reasons. First, Good's alkaline extraction of glycogen destroys most of the glucose in the meat; second, ethanol used to precipitate the glycogen acts as a strong reducing agent toward the copper-tartrate reagent, thus masking the reducing action of glucose.

The method in this paper is a technique to separate the glucose and glycogen in a single meat sample. A non-reducing organic solvent, such as tetrahydrofuran, precipitates glycogen from a raw meat extract. (Tertiary butyl alcohol was also found to act as a non-reducing organic solvent; however, its rate of glycogen precipitation is much slower than that of THF.) The precipitated glycogen is hydrolyzed to glucose and directly determined by copper reduction. The supernatant is deproteinized and its glucose content determined by copper reduction without interference from the organic solvent. The temperature of the meat extracts must be kept low, so that the glucose will not be lost through the formation of Maillard addition products.

EXPERIMENTAL

Reagents

95% ethanol; 66% ethanol; 1.0*M* NaOH, aqueous; 0.18*M* $ZnSO_4$, aqueous; 10% alcoholic KOH (95% ethanol); 0.10*M* $Ba(OH)_2$, aqueous (this solution must be protected from carbon dioxide in the air with a soda-lime tube); 0.05*M* H_2SO_4, aqueous (ca. 5%); aqueous glucose standard, 0.1 mg glucose/ml water; THF glucose standard, 0.1 mg glucose/ml water-THF (17:3 v/v); tetrahydrofuran (THF), anhydrous A. R.; saturated Na_2SO_4 solution, aqueous.

Somogyi's Copper-tartrate reagent. *Solution I:* 12 gm Rochelle salt (sodium potassium tartrate); 24 gm Na_2CO_3, anhydrous; 16 gm $NaHCO_3$; 144 gm Na_2SO_4, anhydrous; diluted to 800 ml with water.

Solution II: 4 gm $CuSO_4 \cdot 5H_2O$; 36 gm Na_2SO_4, anhydrous; diluted to 200 ml with water.

These two solutions are combined to form the copper-tartrate reagent.

Somogyi's Arsenomolybdate reagent. 44.5 gm $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (Ammonium molybdate); 6.0 gm $Na_2HAsO_4 \cdot 7H_2O$ (Sodium acid arsenate); 42.0 ml conc. H_2SO_4.

Extraction

The steps in the following paragraph must be carried out below 5°C. This is done by using an ice bath, or a refrigerated room. A 5–10 gm sample of meat is mixed with 30 ml of water and cut to a fine suspension in an Omni-mixer. The suspension is mechanically agitated for 45 min in a wrist action or linear oscillating shaker bath. Then 150 ml of THF, which has been precooled in an ice-salt bath to about −10°C, is slowly added to the suspension. Efficient stirring and cooling in the ice-salt bath is done so the temperature does not rise above 6°C.

After the addition of THF, the suspension is permitted to stand for a half hour to complete the precipitation of the glycogen. The suspension is then centrifuged for an additional half hour at 480 g's (on the *International* centrifuge model *S B* this corresponds to 2000 rpm at a radius of 10 cm, International Equipment Co., Boston, Mass.) The supernatant is decanted and diluted to 200 ml with water.

Deproteinization

This deproteinization procedure follows the method outlined by Somogyi. A 2-ml aliquot of the supernatant is placed in a 15-ml centrifuge tube. The solution is kept cool in an ice bath while 4 ml of 0.18*M* $ZnSO_4$ and 4 ml of 0.10*M* $Ba(OH)_2$ are added. The resulting mixture is agitated with a vortex mixer and cooled in an ice bath for 5 min. After cooling the mixture is centrifuged long enough to give a clear supernatant. The deproteinized supernatant may now be handled at room temperature.

Copper Reduction

The copper reduction procedure also follows the general method outlined by Somogyi. (The authors attempted to use glucose oxidase as the colorimetric agent for glucose; however it was found to react with dextrins as well as glucose.) A 1-ml aliquot of the deproteinized supernatant is added to a Folin-Wu tube (Folin et al., 1922) along with 1 ml of the copper-tartrate reagent. Two other tubes are also prepared, a blank and a standard. The blank contains 1 ml of copper-tartrate reagent plus 1 ml of a water THF mixture (17:3 v/v). The standard contains 1 ml of copper-tartrate reagent plus 1 ml of the THF glucose standard (0.1 mg glucose/1 ml water-THF 17:3 v/v). The tubes are heated for 10 min in a boiling water bath and then cooled to room temperature. To each tube is added 1 ml of the arsenomolybdate reagent. A colored complex is formed, which is stable for at least 24 hrs. The tubes are agitated with a vortex mixer and then diluted to 25 ml with water. The absorbances of the solutions are measured with a spectrophotometer set at a wavelength of 520 mμ. The amount of glucose present can be calculated with the following equation:

$$\text{mg glucose} = A_{\text{sample}} \times \frac{0.1 \text{ mg}}{A_{\text{std}}} \times (200 \times 5)$$

where

A_{sample} = absorbance of sample
A_{std} = absorbance of standard
(200×5) = dilution factors

Glycogen purification

The residue precipitated by THF is di-

Table 1—Comparison of new method with accepted methods.

Sample number	Somogyi's method % glucose	Good's method % glycogen	New method % glucose	New method % glycogen	Comparison New / Somogyi × 100 glucose	Comparison New / Good × 100 glycogen
1	0.117	0.388	0.125	0.427	108.7	110.2
2	0.146	0.467	0.145	0.456	99.0	97.7
3	0.202	0.535	0.195	0.501	96.2	93.7
4	0.173	0.459	0.194	0.460	112.3	100.4
5	0.121	0.380	0.116	0.370	95.6	102.9
6	0.138	0.268	0.130	0.251	93.2	93.4
7	0.150	0.276	0.150	0.295	100.0	107.0
8	0.105	0.295	0.106	0.296	100.4	100.5
9	0.122	0.385	0.119	0.373	96.8	96.7
10	0.201	0.496	0.186	0.487	92.5	98.0
11	0.107	0.540	0.115	0.625	107.1	115.8
				Mean	100.1	101.5
				Standard error	2.0	2.1

Table 2—Glucose recoveries using new method.

Sample number	Glucose content initial analysis (mg)	Amount of glucose added (mg)	Glucose content final analysis (mg)	% Recovery
12	40.79	0	40.79	100.0
13	28.96	10	36.80	93.2
14	34.43	25	54.98	92.6
15	25.96	50	74.77	98.5
16	28.48	70	97.12	98.6
17	24.58	90	110.92	96.8

Table 3—Glycogen recoveries using new method.

Sample number	Glycogen content initial analysis (mg)	Amount of glycogen added (mg)	Glycogen content final analysis (mg)	% Recovery
12	43.44	0	43.44	100.0
13	30.84	10	39.26	95.3
14	36.68	25	63.20	102.4
15	27.63	50	73.63	94.9
16	30.33	70	91.00	90.7
17	26.19	90	112.94	97.2

gested in 25 ml of 10% alcoholic KOH at 75°C for 10–20 min. This is accompanied by vigorous stirring with a glass rod. The digestion is terminated when all of the residue has been dissolved by the alcoholic KOH. Then 5 ml of saturated sodium sulfate solution, followed by 100 ml of 95% ethanol is added to the solution. The resulting mixture is centrifuged for $^1/_2$ hr at 480 *g*. The supernatant is discarded, and the precipitate is washed three times with 66% ethanol and once with ether.

Glycogen hydrolysis

Approximately 35 ml of 0.50*M* H_2SO_4 is added to the purified glycogen. This mixture is refluxed for at least 5 hr. Then the acidic solution is neutralized with 1.0*M* NaOH to a pH of 6.5–7.0. Efficient stirring must accompany the neutralization to avoid local concentrations of NaOH which destroy the glucose. The neutralized glucose solution is diluted to 200 ml with water. This solution is analyzed by the copper reduction method, with the exception that the blank and the standard do not contain THF.

DISCUSSION & RESULTS

TWO CALIBRATION curves were plotted, one for glucose standards dissolved in pure water and one for glucose standards dissolved in a water-THF mixture (17:3 v/v). Both curves are linear, which indicates that Beer's law is valid for this method and that the absorbance is directly proportional to the glucose concentration.

Meat samples were analyzed in triplicate for glucose by Somogyi's method and for glycogen by Good's method. The same samples were also analyzed by the new method. The results of the new and standard methods of analysis are summarized in Table 1.

Table 4—Glucose loss due to warming of meat extracts (new method).

Sample number	Extraction temp. (4–5°C) Glucose content (mg)	Extraction temp. (15–20°C) Glucose content (mg)	% Glucose lost
18	1.15	0.97	25.6
19	3.04	2.13	30.1
20	2.60	2.19	25.9

A series of recoveries was run in which varying amounts of glucose and glycogen were added directly to the raw meat extracts. These extracts were then analyzed by the new method. Also, an initial analysis, using the new method, was run on samples of the same series to which no additional carbohydrates had been added. The results are summarized in Tables 2 and 3. This data was treated with the least squares adjustment to obtain plots of percent recovery versus amount of carbohydrate added for both glucose and glycogen. The glucose plot was linear with a slope of 0.07. The glycogen plot was also linear with a slope of −0.05. These slopes are not significantly different from zero which indicates that the lines are essentially horizontal.

The authors made a study of the effect of temperature on the new method. Table 4 shows the results of an analysis in which the temperature of the meat extract was raised from 5°C to 15–20°C for 10 min. The results are compared with a similar analysis run at the prescribed temperature (4–5°C). The last column shows the amount of glucose lost in increasing the temperature of the extract. The loss of glucose is attributed to the fact that at temperatures above 5°C glucose forms Maillard reaction products with amino acids (Chichester et al., 1952). The glucose content is significantly lowered with an increase in temperature.

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Nutritive Value of 1,2-Dichloroethane-Extracted Fish Protein Concentrate

SUMMARY—Studies were conducted on the nutritive value of fish protein concentrate (FPC) preparations produced by extracting cod fillet with 1,2-dichloroethane (DCE). These were fed to immature rats at either a 20% dietary protein level or at a 10% protein level in conjunction with 10% casein protein. Findings were compared to those from immature rats fed diets containing 10 or 20% casein protein. Nutritive value of the FPC preparations was dependent on the temperature and length of time at which extraction occurred. Rats fed diets containing FPC extracted at 65° ± 2°C or 40° ± 2°C for 24 hr or at a temperature of 83°C for 6 hr or 3 hr and vacuum dried without steaming were normal in appearance and exhibited increments in body weight and feed efficiency comparable to those of rats fed similar diets containing 20% casein protein. FPC prepared under similar conditions except that it was extracted for 24 hr at 83°C without replacing evaporated solvent during the last 6 hr of extraction was toxic. Subjecting the latter to vacuum steaming for 45 min at 10 in. of vacuum eliminated its toxic properties as determined in a 7-day feeding test, although some impairment of its nutritional value was still manifest. Cod fillet prepared by the same procedure as the toxic material except that evaporated solvent was replaced at 2–3 hr intervals throughout the entire 24-hr period of extraction was non-toxic in a 7-day feeding test and differed only slightly from casein in nutritive value.

INTRODUCTION

MUNRO et al. (1967) reported that lyophilized cod fillets extracted for 24 hr with 1,2-dichloroethane (DCE) were toxic when fed to immature rats. Animals fed this material at a 50% dietary protein level lost weight and died within 7 days. In contrast, rats fed diets containing a similar amount of non-extracted lyophilized cod fillet or cod fillet extracted for 24 hr with isopropyl alcohol remained alive, gained weight and appeared normal. The present report indicates that the toxicity of DCE-extracted cod fillet is dependent on the temperature and length of extraction time, and that it is possible to prepare DCE-extracted fish protein concentrate (FPC) that is non-toxic and of high nutritional value when fed as the sole source of protein to immature rats.

EXPERIMENTAL

FPC PREPARATIONS were prepared from fresh cod fillet which was ground through a commercial meat grinder. Ground fillet was immediately placed in a steam jacketed kettle with a lid and DCE was added in the ratio of 15 gal DCE to 12.5 lb ground fillet. The FPC preparations were extracted at 83°C for periods of 24, 6 and 3 hr and at 65° ± 2°C and 40° ± 2°C for 24 hr. A water-DCE mixture azeotropes at 71.5°C. As long as sufficient amounts of water were present in the cod fillet this temperature was maintained in the preparations extracted at 83°C; but as water evaporated and the anhydrous state was approached, temperature rose rapidly to 83°C, the boiling point of DCE. The latter temperature was attained within 30 min of the start of extraction. The periods cited for preparations extracted at 83°C (3, 6 and 24 hr) include time that extraction was occurring at azeotropic temperature. In the preparations extracted at 83°C considerable amounts of solvent were lost by evaporation. Additional amounts of DCE were accordingly added at 2 to 3 hr intervals to replace that lost by evaporation. However, solvent lost by evaporation from Sample A, extracted at 83°C for 24 hr, was not replaced after the 18th hour of extraction.

At the conclusion of the extraction period, the ground fish was removed from the kettle and pressed free of DCE, water and fatty material. The moist, defatted material was spread on trays in a 1/2 in. layer, placed in a vacuum oven and dried under 29 in. of vacuum at a temperature of 100°C for 18 hr. Subsequent to drying, all preparations were ground into a fine powder in a 1 gal Waring Blendor and passed through a 20 mesh screen. Approximately 10% of the material did not pass through the screen and was discarded. Only that portion which passed through the screen was employed in the feeding tests.

The FPC preparations were analyzed for their protein and residual DCE content, and their nutritive value as sources of dietary protein was determined as indicated below.

Male rats of the Long-Evans strain, 24–26 days of age, were fed purified diets containing 10% cottonseed oil, 5% salt mixture (Wesson Modification of Osborne-Mendel Salt Mixture, General Biochemicals, Chagrin Falls, Ohio) and the following vitamins per kg of ration: thiamine hydrochloride, 10 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 10 mg; calcium pantothenate, 60 mg; nicotinic acid, 100 mg; ascorbic acid, 200 mg; para-aminobenzoic acid, 200 mg; inositol, 400 mg; biotin, 1 mg; folic acid, 5 mg; vitamin B_{12}, 150 μg; 2-methyl, 1-4 naphthoquinone, 5 mg; choline chloride, 2 g; vitamin A, 5000 U.S.P. units; vitamin D_2, 500 U.S.P. units; and alpha tocopheryl acetate, 100 mg.

Diets were prepared containing 10% casein protein (Vitamin-Free Test Casein, General Biochemicals, Chagrin Falls, Ohio), 20% protein as either casein or FPC, and 20% protein as combinations of casein and FPC with half the protein provided by casein and the remainder by FPC. The balance of each diet consisted of sucrose.

Animals were placed in individual metal cages with raised screen bottoms and were provided the test diets and water ad libitum. The rats were kept in an air-conditioned room maintained at 72° ± 3°F. Animals were fed daily and all food not consumed 24 hr after feeding was discarded. Food consumption was determined daily for each rat. The duration of the experimental period was 2 weeks in Experiments 1, 2 and 3, and 1 week in Experiment 4. Animals were weighed weekly during the course of the experiment. Data were obtained on the average increments in body weight and feed efficiency (judged by average weight gain/g of food ingested) on the various diets employed.

RESULTS

Experiment 1

Tests were conducted to determine the effects of extraction temperature on the nutritive value of FPC prepared by extracting fresh ground cod fillet for 24 hr with DCE. Forty-eight male rats with an average body weight of 69.1 g (range 64–74 g) were divided into eight groups of six animals each and were fed the diets indicated in Table 1.

Findings confirmed earlier observations that a 10% casein protein diet is inadequate for optimal growth and feed efficiency in the immature rat. Rats fed the 10% casein protein diet gained 29.8 g after 14 days of feeding and had a feed efficiency of 0.213 g. Supplementing the above diet with an additional 10% casein protein increased weight gain after 14 days to 88.2 g and increased feed efficiency to 0.458 g.

Rats fed FPC Sample C (extracted with DCE for 24 hr at 40° ± 2°C) at a 20% dietary protein level or at a 10% level in conjunction with 10% casein protein exhibited increments in body weight and

Table 1—Effects of processing temperature on nutritive value of FPC extracted for 24 hr with DCE (6 rats per group).

Dietary group	Initial body wt. (g)	Average gain (g) in body wt. after 7 days	Average gain (g) in body wt. after 14 days	Average food consumption per rat for 14-day period (g)	Average gain in body wt. per g of food eaten[1] (g)	Residual DCE content of FPC preparations (ppm)
10% protein as casein[2]	69.2	10.5	29.8	139.9	0.213	—
20% protein as casein	69.0	39.8	88.2	192.7	0.458	—
10% protein as casein + 10% protein as FPC Sample A[3]	69.0	−13.7	−12.0 (5)	76.7	—	30,000
20% protein as FPC Sample A	69.2	−21.0 (1)	—	—	—	30,000
10% protein as casein + 10% protein as FPC Sample B[4]	69.1	38.2	84.7	197.1	0.430	2,100
20% protein as FPC Sample B	69.0	36.9	83.0	189.0	0.439	2,100
10% protein as casein + 10% protein as FPC Sample C[5]	69.2	42.8	88.8	188.9	0.470	17
20% protein as FPC Sample C	69.1	41.6	88.5	186.5	0.475	17

The values in parentheses indicate the number of animals which survived and on which averages were based when this number was less than the original number per group.

[1] This value constitutes a better indicator of the nutritional value of a diet than that for Protein Efficiency Ratio (PER) (wt. gain/protein intake). The latter can readily be calculated from values in the table by multiplying such values by 5 in the case of diets with a protein content of 20% and by 10 in the case of diets with a protein content of 10%.

[2] The protein content of this material was 85.19% (13.63% N × 6.25).

[3] FPC Sample A was extracted with DCE for 24 hr at a temperature of 83°C. It had a protein content of 86.75% (13.88% N × 6.25).

[4] FPC sample B was extracted with DCE for 24 hr at a temperature of 65° ± 2°C. It had a protein content of 95.00% (15.20% N × 6.25).

[5] FPC Sample C was extracted with DCE for 24 hr at a temperature of 40° ± 2°C. It had a protein content of 95.63% (15.30% N × 6.25).

Table 2—Effects of length of time that extraction occurs on the nutritive value of FPC extracted with DCE at a temperature of 83° × 2°C (6 rats per group).

Dietary group	Initial body wt. (g)	Average gain (g) in body wt. after 7 days	Average gain (g) in body wt. after 14 days	Average food consumption per rat for 14-day period (g)	Average gain in body wt. per g of food eaten (g)	Residual DCE content of FPC preparations (ppm)
20% protein as casein	62.0	39.2	87.0	180.6	0.482	—
20% protein as FPC Sample A[1]	62.2	—[4]	—	—	—	30,000
20% protein as FPC Sample D[2]	62.0	37.1	84.5	177.8	0.475	995
20% protein as FPC Sample E[3]	62.1	38.2	90.5	189.2	0.478	40

[1] See footnote 3, Table 1.

[2] FPC Sample D was extracted with DCE for 6 hr at a temperature of 83°C. It had a protein content of 93.31% (14.61% N × 6.25).

[3] FPC Sample E was extracted with DCE for 3 hr at a temperature of 83°C. It had a protein content of 93.31% (14.93% N × 6.25).

[4] All rats in this group died during the first week of feeding.

Table 3—Effects of graded levels of DCE on the weight increment and efficiency of food utilization of immature rats fed a purified ration containing casein as the source of dietary protein (6 rats per group).

Dietary group	Initial body wt. (g)	Average gain (g) in body wt. after 7 days	Average gain (g) in body wt. after 14 days	Average food consumption per rat for 14-day period (g)	Average gain in body wt. per g of food eaten (g)
20% protein as casein	72.8	54.8	106.8	209.7	0.509
20% protein as casein plus following supplements:					
470 mg DCE per kg of diet	72.8	52.6	104.2	205.8	0.506
940 mg DCE per kg of diet	73.0	52.4	102.6	217.1	0.473
1.88 g DCE per kg of diet	73.0	52.9	105.3	231.5	0.455
3.525 g DCE per kg of diet	73.0	47.4	95.6	216.5	0.442
7.05 g DCE per kg of diet	73.0	47.8	93.8	208.3	0.450

feed efficiency equal to those of rats fed the diet containing 20% casein protein.

Rats fed FPC Sample B (extracted with DCE for 24 hr at 65° ± 2°C) at a 20% dietary protein level or at a 10% level in conjunction with 10% casein protein exhibited increments in body weight and feed efficiency which were only slightly lower than those of rats fed the 20% casein protein diet.

Rats fed FPC Sample A (extracted with DCE for 24 hr at 80°C) at a 20% dietary protein level, however, lost weight from the start of feeding and died within 8 days. Rats fed FPC Sample A at a 10% level in conjunction with 10% casein protein also lost weight during the course of the experiment although 5 of the 6 rats survived. The weight loss was associated with a marked reduction in food intake compared to that of rats fed the other diets tested. Results are summarized in Table 1.

Findings obtained with FPC Sample A are in agreement with those reported by Munro et al. (1967) who observed that lyophilized cod fillets extracted for 24 hr with DCE were toxic when fed to immature rats. No evidence of toxicity was observed, however, in rats fed diets containing FPC Samples B and C, extracted for 24 hr with DCE at 65° ± 2°C and 40° ± 2°C, respectively.

Residual DCE content was determined according to methods prescribed by the Food and Drug Administration (1968). Significant differences were observed in the residual DCE content of FPC Samples A, B and C despite the fact that all 3 samples were dried in a similar manner (spread on trays in a 1/2 in. layer and vacuum dried under 29 in. of vacuum at 100°C for 18 hr). Findings indicate that toxic FPC Sample A had a residual DCE content of 30.000 ppm whereas non-toxic FPC Samples B and C had residual DCE contents of 2100 and 17 ppm respectively.

Experiment 2

Tests were conducted to determine the effects of length of extraction time on the nutritive value of FPC prepared by extracting fresh ground cod fillet with DCE at 83°C. Twenty-four male rats with an average weight of 62.1 g (range 58–66 g) were divided into 4 groups of 6 animals each and fed the diets indicated in Table 2.

As in Experiment 1, rats fed a diet containing 20% protein from FPC Sample A (extracted with DCE at 83°C for 24 hr) lost weight and died within 7 days. Rats fed diets containing 20% protein from FPC Samples D and E (extracted with DCE at 83°C for 6 and 3 hr respectively) exhibited increments in body weight and feed efficiency comparable to those of rats fed a 20% casein protein diet. No evidence of toxicity was observed in rats

fed diets containing FPC Samples D and E. Results are summarized in Table 2.

As in Experiment 1 significant differences were observed with respect to residual DCE content despite the fact that all FPC preparations were dried in a similar manner. In contrast to the residual DCE content of 30,000 ppm in FPC Sample A, non-toxic FPC Samples D and E had residual DCE contents of 995 and 40 ppm respectively.

Experiment 3

Findings in Experiments 1 and 2 indicate that toxic FPC Sample A had a residual DCE content of 30,000 ppm whereas non-toxic FPC Samples B, C, D and E had residual DCE contents of 2100 ppm or less. These findings raised the question as to whether DCE present in FPC Sample A was responsible for its toxic effect. Studies were undertaken to determine the effects of graded levels of DCE incorporated in a purified ration containing 20% casein protein. The amounts of DCE added/kg of diet are indicated in Table 3. The highest level (7.05 g DCE/kg diet) was the amount of residual DCE present in the diet containing 20% FPC Sample A.

In contrast to the findings obtained in Experiments 1 and 2 with FPC Sample A, all rats fed the diet containing 7.05 g DCE/kg of diet survived, gained weight and appeared normal although their increments in body weight and feed efficiency were slightly less than those of rats fed a similar diet with DCE omitted. Results are summarized in Table 3.

The above findings suggest that the toxicity of FPC Sample A was due to some factor other than its residual DCE content per se. It is possible that in FPC Sample A the residual DCE was bound in such a manner as to form a toxic factor or factors which either did not occur or was present in minimal amounts in the other FPC preparations. Another possibility is that DCE may be toxic or form a toxic substance when added to a diet containing FPC derived from cod fillet which might not occur in a diet containing casein as the protein source.

The question also occurs as to what extent adding 7.05 g DCE to the casein-containing diet simulates conditions in the diet containing FPC Sample A. It is possible that on the former diet considerably more DCE was lost by evaporation during the 24-hr period between feedings than was the case for the diet containing FPC Sample A. If such were the case, then animals ingesting the diet containing the highest level of added DCE may actually have consumed a far smaller amount of this solvent than those fed diets containing FPC Sample A.

Experiment 4

Tests were conducted to determine the effects of steaming during vacuum drying and the effects of replacing solvent lost by evaporation during extraction on the toxicity and nutritive value of FPC prepared by extracting ground fillet with DCE for 24 hr at 83°C. Forty-eight male rats with an average body weight of 67.1 g (range 63–71 g) were divided into 8 groups of 6 animals each and fed the diets indicated in Table 4.

Table 4—Effects of steaming during vacuum drying and of replacing solvent lost by evaporation throughout the extraction period on the nutritive value of FPC extracted for 24 hr with DCE at a temperature of 83°C (6 rats per group).

Dietary group	Initial body wt. (g)	Average gain in body wt. after 7 days of feeding[1] (g)	Average food consumption per rat for 7-day period (g)	Average gain in body wt. per g of food eaten (g)	Residual DCE content of FPC preparations (ppm)
10% protein as casein	67.0	13.5	65.7	0.205	—
20% protein as casein	66.8	39.5	81.0	0.488	—
10% protein as casein + 10% protein as FPC Sample A[2]	67.0	−12.6	36.4	—	30,000
20% protein as FPC Sample A	67.2	—[4]	—	—	30,000
10% protein as casein + 10% protein as FPC Sample F[3]	67.0	28.5	69.8	0.408	13
20% protein as FPC Sample F	67.2	8.0	47.1	0.170	13
10% protein as casein + 10% protein as FPC Sample G[4]	67.2	37.0	78.1	0.474	28
20% protein as FPC Sample G	67.0	35.3	79.8	0.442	28

[1] The experiment was terminated after 7 days of feeding due to insufficient amounts of FPC Sample F to continue further testing.

[2] See footnote 3, Table 1.

[3] FPC Sample F was prepared by subjecting FPC Sample A to vacuum steaming for 45 min at 10 in. of vacuum.

[4] FPC Sample G was extracted with DCE for 24 hrs at a temperature of 83°C with the solvent lost by evaporation replaced at 2 to 3 hr intervals throughout the entire 24 hr extraction period. It was vacuum dried without steaming in the same manner as FPC Sample A. It had a protein content of 93.94% (15.03% N × 6.25).

Findings in Experiments 1 and 2 indicate that toxic FPC Sample A had a residual DCE content of 30,000 ppm while other FPC preparations extracted for a similar length of time at lower temperatures or at the same temperature for shorter periods were non-toxic and had residual DCE contents of 2100 ppm or less. Since E. Levin and V. K. Collins (unpublished data, 1967) observed that steaming during vacuum drying resulted in a significant reduction in residual DCE of DCE-extracted FPC, studies were conducted to determine the effects of vacuum steaming FPC Sample A and comparing the response of rats fed the steamed material (FPC Sample F) at a 20% dietary protein level and at a 10% protein level in conjunction with 10% casein protein to that of rats fed similar diets containing FPC Sample A. Vacuum steaming FPC Sample A for 45 min at 10 in. of vacuum reduced the residual DCE content from 30,000 to 13 ppm.

In agreement with findings in Experiments 1 and 2, rats fed diets containing FPC Sample A at either a 20% protein level or at a 10% protein level in conjunction with 10% casein protein lost weight. All animals receiving the former diet died within 7 days. In contrast, all rats fed similar diets containing FPC Sample F (vacuum steamed) survived and gained weight.

The increments in body weight and feed efficiency were significantly less for rats fed the diet containing 20% FPC Sample F protein than for those fed the 20% casein protein diet. However, both increments were greater for rats fed the diet containing 10% casein protein plus 10% FPC Sample F protein than for rats fed the diet containing 10% casein protein alone. Increments in body weight and feed efficiency on the 10% casein protein plus 10% FPC Sample F protein diet were less, however, than those of rats fed the 20% casein protein diet.

These findings indicate that the nutritional value of FPC Sample F as a protein source was less than that of casein. It did have value when fed as a supplement to a diet containing a suboptimal amount of casein protein, however. Although FPC Sample F had an impaired nutritional value compared to FPC Samples B, C, D and E, it was non-toxic when fed to immature rats in a 7-day feeding test. This is indicated by the normal appearance, increased weight gain and increased feed efficiency of rats fed this material as a supplement to a 10% casein protein diet. It is apparent from these findings that vacuum steaming FPC Sample A resulted not only in a striking reduction of its residual DCE content (from 30,000 to 13 ppm) but also in elimination of its toxic effect.

In FPC preparations extracted at 83°C considerable amounts of solvent were lost by evaporation. In the case of FPC Samples D and E this solvent was replaced at

2–3 hr intervals by adding additional amounts of DCE so that the amount of solvent present at the conclusion of the extraction period differed little if at all from that initially employed.

In the case of FPC Sample A, however, the evaporated solvent was replaced during only the first 18 hr of extraction. Inadvertently, no DCE was added during the final 6 hr and the amount of solvent at the conclusion of the 24-hr extraction period was consequently less than one-fourth that originally present. The importance of maintaining the initial volume of solvent was demonstrated in the following experiment.

Since FPC Sample A differed from FPC Samples D and E not only in the length of extraction but also in the amount of solvent at the termination of the extraction period, studies were conducted to determine the effects of replacing evaporated solvent throughout the entire 24-hr extraction period on the nutritive value of the resulting FPC preparation. The experimental procedure for preparing the latter was identical to that employed in preparing FPC Sample A except that DCE was added at 2–3 hr intervals throughout the 24-hr extraction period. This preparation was vacuum dried without steaming in the same manner as FPC Sample A. The residual DCE content of this preparation (FPC Sample G) was 28 ppm in contrast to the 30,000 ppm in FPC Sample A.

In contrast to findings obtained with FPC Sample A, all rats fed diets containing FPC Sample G survived and exhibited increments in body weight and feed efficiency that were only slightly less than those of rats fed the 20% casein protein diet. Results are summarized in Table 4. Findings indicate that when evaporated solvent is replaced at 2–3 hr intervals, it is possible to prepare FPC which, when extracted for 24 hr with DCE at a temperature of 83°C and dried under 29 in. of vacuum at 100°C for 18 hr without steaming, is non-toxic, low in residual DCE content and high in nutritive value.

DISCUSSION

MORRISON et al. (1965) reported that extracting freeze-dried cod fillet with DCE destroyed cystine and histidine and interfered with the in vitro enzymatic release of cystine, histidine and methionine. These effects were time and pH dependent. They were particularly marked after 8 and 16 hr extraction and under alkaline conditions. At a pH of 4.5, little or no destruction or interference with in vitro enzymatic release of these amino acids occurred.

More recently Munro et al. (1967) reported that lyophilized cod fillets extracted for 24 hr with DCE and subsequently heated for 2 hr at 60°C in a vacuum oven to remove residual solvent were toxic when fed as the sole protein source to immature rats. The toxic factor or factors could be removed at least in part by methanol extraction. Rats fed the methanol-extracted material, however, still failed to gain weight even when it was supplemented with cystine and histidine. As pointed out by Munro et al. (1967) these findings suggest that methanol did not remove all of the toxic material from the DCE-extracted fish. The failure of rats fed the methanol-extracted material even when supplemented with cystine and histidine, however, might also have been due to the destruction or non-availability of essential amino acids other than the above.

No data on the residual DCE content of the above preparations were presented although Morrison et al. (1962) have stressed that the nutritional value of FPC preparations may be dependent on the degree to which toxic solvent residues are removed. They have pointed out that in some cases solvent may be so tightly bound that it is not readily removed by heating for prolonged periods of time and this was confirmed in the the present study.

Findings indicate that a FPC preparation (FPC Sample A) which was prepared by extracting fresh cod fillet with DCE for 24 hr at a temperature of 83°C and which was dried under 29 in. of vacuum at a temperature of 100°C for 18 hr without steaming was highly toxic when fed to immature rats and that this toxicity was associated with a residual DCE content of 30,000 ppm. When DCE was added to fresh cod fillet and the mixture was heated, water in the fillet began to evaporate as soon as the azeotropic temperature of water-DCE (i.e., 71.5°C) was reached. Within 30 min sufficient amounts of water had evaporated to result in a temperature rise to 83°C, the boiling point of DCE.

It is possible that when lyophilized cod fillet or cod fillet whose moisture content has been azeotropically reduced is extracted with DCE for 24 hr the latter is bound in a form that is not readily removed in the absence of vacuum steaming. The lower levels of residual DCE in FPC extracted for 24 hr at a temperature of 65° ± 2°C or 40° ± 2°C might be accounted for on the basis that at the latter temperature sufficient amounts of moisture were retained in the cod fillet to prevent binding of DCE.

Although data were obtained which suggest that the toxicity of FPC Sample A was due to some factor other than residual DCE content per se, the possibility that residual DCE was bound in a manner to form a toxic factor or factors was not excluded. When the residual DCE content of this material was subsequently reduced to 13 ppm by vacuum steaming for 45 min at 10 in. of vacuum, its toxic properties as determined in a 7-day feeding test were eliminated although some impairment in its nutritional value was still manifest. It is of interest that the beneficial effect of steaming on FPC preparations was also observed by Morrison (1963) who found that steaming a sample of fish flour whose sulfur-containing amino acids were unavailable as determined by biological evaluation increased its ability to supplement a methione deficient diet.

Present findings indicate that the nutritive value of DCE-extracted cod fillet was dependent on the temperature and length of time at which extraction occurred. Rats fed diets containing FPC which was extracted at 65° ± 2°C or 40° ± 2°C for 24 hr, or at a temperature of 83°C for 6 hr or 3 hr and which were dried under vacuum without steaming were normal in appearance and exhibited an increment in body weight and efficiency of food utilization that were comparable to that of rats fed similar diets containing 20% casein protein.

In contrast, FPC prepared under similar conditions except that it was extracted for 24 hr at a temperature of 83°C and evaporated solvent was not replaced during the last 6 hr of extraction was toxic. Subjecting the latter preparation to vacuum steaming for 45 min at 10 in. of vacuum eliminated toxic properties as determined in a 7-day feeding test, although some impairment of its nutritional value was still manifest. Cod fillet which was prepared by the same procedure as the toxic material except that solvent lost by evaporation was replaced at 2–3 hr intervals throughout the 24-hr extraction period was non-toxic in a 7 day feeding test and differed only slightly from casein in nutritive value.

No data are available to account for the difference in toxicity between the latter preparation (FPC Sample G) which was non-toxic and the toxic effects obtained with FPC Sample A which was processed in an identical manner except that evaporated solvent was not replaced during the last 6 hr of the extraction period. Water was present in the DCE employed at a level of 800 ppm. The question arises as to whether water present in the DCE which was added during the last 6 hr of extraction in the preparation of FPC Sample G might have contributed to the diverse effects obtained.

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Quantitative Determination of Formic, Acetic, Propionic and Butyric Acids in Frozen Whole Eggs by Gas-Liquid Chromatography

SUMMARY—Whole eggs containing known amounts of formic, acetic, propionic and butyric (C_1–C_4) acids were evaluated using gas-liquid chromatography (GLC) and AOAC (1960) procedures. Acetic, propionic and butyric acids were recovered from whole egg samples and chromatographed as the acids per se, along with an internal standard. Formic and acetic acids were recovered from whole egg samples and chromatographed as their butyl ester derivatives without an internal standard. Quantitation of butyl formate and butyl acetate was accomplished by comparing the chromatogram peak heights of the butyl esters recovered from the egg samples with the chromatogram peak heights of a known concentration of butyl ester standards. The recovery of acids from whole egg samples by GLC procedures was as accurate, and generally less variable, than by AOAC (1960) procedures. The percentage recovery of acids from whole egg samples using GLC procedures depended on the concentrations of the acids present and was as follows: acetic acid varied from 114 to 100% (low to high concentration); propionic acid varied from 101 to 100% (low to high concentration); and butyric acid varied from 103 to 99% (low to high concentration). The recovery of formic acid as its butyl ester derivative varied from 104 to 96% (low to high concentration), while the recovery of acetic acid as its butyl ester derivative varied from 102 to 98% (low to high concentration).

INTRODUCTION

SEVERAL METHODS have been proposed for the determination of formic acid in biological materials. Fincke (1913) developed a gravimetric procedure for the determination of formic acid based on its reaction with mercuric chloride. Hanak et al. (1930) reported the development of a permanganate colorimetric method for the determination of formic acid. A relatively simple mercuric chloride volumetric method for the estimation of formic and acetic acids was developed by Fuchs (1929). Grant (1948) proposed a colorimetric method for the microdetermination of formic acid based on the reduction of the acid to formaldehyde.

Dyer (1917) proposed the original procedure for the separation, identification and determination of volatile fatty acids by steam distillation. This procedure is based on the fact that under specified conditions the volatile acids distill at a constant rate, and in a mixture of volatile acids, each acid distills as if it were present singly. This procedure has been modified somewhat by several workers (Werkman, 1930; Osborn et al., 1936; and Hillig et al., 1942).

Ramsey et al. (1945) and Ramsey (1963) proposed methods for the separation and identification of micro-amounts of formic, acetic, propionic and butyric acids (C_1–C_4). The volatile acids were separated on a silicic acid chromatographic partition column, and the C_1–C_4 acids were positively identified by microscopic examination of their characteristic crystalline mercurous salts.

Attempts have also been made to separate and identify the short-chain volatile acids using paper chromatography (Reid et al., 1952; Stark et al., 1951; Kennedy et al., 1951; and Isherwood et al., 1953).

Shelley et al. (1963) described a procedure whereby the volatile short-chain fatty acids (C_1–C_4) present in frozen eggs were quantitatively determined, using GLC. A similar method was also used by Grey et al. (1966) for the quantitative determination of formic, acetic and propionic acids from microbial fermentations.

Both Shelley et al. (1963) and Grey et al. (1966) used GLC units equipped with S^{90} detectors. Since a gas-liquid chromatograph having this type of detector was not available, the study was initiated to determine if the C_1–C_4 acids present in frozen whole eggs could be quantitatively determined using a flame-ionization detector.

PROCEDURES

Apparatus and general procedures

Gas-liquid chromatograph. An F & M Scientific Company Model 810 dual column flame ionization instrument equipped with a Model 9294 N Honeywell recorder was used for all GLC analyses in this study.

Steam distillation assembly. This assembly consisted of a 2,000-ml round bottom flask which served as a steam generator and was connected to a two-neck 1,000-ml round bottom distillation flask by means of an arrangement of glass and rubber tubing and neoprene stoppers. The steam entered the distillation flask through the center neck, and the volatile products in the sample were allowed to escape through a Vigreux fractioning column connected to the other neck. The volatile products, after passage through the Vigreux column, were condensed in a Graham coil condenser and subsequently collected in a 2,000-ml graduated cylinder. Heat for both the steam generator and the distillation flask was supplied by means of individual heating mantles connected to variable voltage transformers. Figure 1 shows the details of this apparatus.

Preparation of whole egg-acid mixtures

Newly laid eggs were obtained from the University Poultry Farm. The eggs were broken and blended in a 1.5-gal stainless steel Waring-Blendor jar for 15 sec at low speed, transferred to a 30-gal stainless steel multiple paddle mixer and mixed for 15 min.

The blended whole eggs were divided into 5,000-g lots and prepared for analyses by

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Table 1—Concentration of acids in whole egg mixtures, mg/100g egg.

	Group 1			Group 2	
Sample	Acetic	Propionic	Butyric	Formic	Acetic
1	1.17	1.38	1.70	1.25	1.15
2	3.17	3.72	4.59	2.47	3.30
3	6.34	7.44	9.29	4.94	6.60
4	12.67	14.87	18.37	9.88	13.20
5				24.76	33.32

adding increasing aliquots of previously prepared $N/10$ standard solutions of acetic, propionic and butyric acids to specified lots and formic and acetic acids to other specified lots. Each mixture was then blended for 15 sec at low speed. From each 5,000-g acid-egg mixture, 20 subsamples of 225 g each were placed into pint plastic containers and frozen and held to −10°C until analyzed. The concentration of acids in egg mixtures is shown in Table 1.

Preparation of egg samples for AOAC analysis

Triplicate samples of eggs containing formic, acetic, propionic and butyric acids from the same group of eggs analyzed by GLC were analyzed according to AOAC (1960) procedures 16.035–16.036 and 18.019–18.022, respectively. The C_2–C_4 acid content of the eggs was quantitated by titrating each acid with sodium hydroxide following elution from a silicic acid column. The formic acid content was quantitated gravimetrically by its reaction with mercuric chloride.

Column preparation and operation

Twenty grams of high-temperature stabilized ethylene glycol adipate (Analabs) were dissolved in acetone. Eighty grams of 120/130 mesh Anakrom ABS (Analabs) were added to the dissolved solution and mixed with the aid of a magnetic stirrer. The acetone was removed in a rotating evaporator at reduced pressure, using a water bath at 45°C. The packing material was further dried in an air oven for 12 hr at 100°C.

The columns were packed using a vacuum and Vibragraver tool to evenly distribute the packing material within the column. The packed columns were installed in the chromatograph and conditioned for 24 hr at 175°C. Nitrogen was used as the conditioning gas at a flow rate of 50 ml/min. The operating parameters for the gas-liquid chromatograph are shown in Table 2.

Preparation of internal standard

Approximately 3 g of butyl octanoate (K & K Labs) were accurately weighed into a 25-ml glass-stoppered volumetric flask and made to volume with acetone. This solution was designated as the "stock solution." A 1:10 dilution of the stock solution was prepared, using acetone as the diluent and designated as the "diluted stock solution."

Dichloracetic acid solution

Twenty-five grams of redistilled dichloroacetic acid (Fisher Scientific Company, certified reagent grade) were accurately weighed into a 200-ml volumetric flask, diluted to the mark with acetone, and tightly stoppered.

Calibration of chromatograph

Approximately 1 g each of acetic, propionic and butyric acids and butyl octanoate (K & K Labs) were accurately weighed into the same 25-ml volumetric flask, brought to volume with acetone and designated as the stock solution. A 1:10 dilution of the stock solution was prepared by pipetting 2.5 ml of the stock mixture into a 25-ml volumetric flask and making to volume. Various volumes of the 1:10 dilution mixture of the standard acids were pipetted into a 5-ml volumetric flask to provide a range of concentration for each acid at a given sensitivity. The peak heights of acetic, propionic and butyric acids and butyl octanoate were measured following five replicate one μl-injections of each concentration into the GLC.

Using the calculation outlined by Shelley et al. (1963), the detector response value, R, of the C_2–C_4 acids relative to butyl octanoate was calculated using the formula: $R_A = (h_A)\ (c_{IS})/(c_A)\ (h_{IS})$, where h_A and h_{IS} are the peak heights of the acid and butyl octanoate, respectively, and c_A and c_{IS} are the corresponding concentrations (mg per 5-ml acetone solution).

Acid recoveries by steam distillation

One hundred milliliters each of $N/10$ standard formic, acetic, propionic and butyric acid solutions were pipetted into individual 200-ml volumetric flasks and brought to volume with water. Three 10-ml aliquots of each acid were removed by pipette, placed in a 125-ml Erlenmeyer flask with 10 ml of water, and titrated with $N/100$ sodium hydroxide to a phenolphthalein end point.

Ten milliliter aliquots of each acid were pipetted into the distillation flask of the steam distillation assembly. Four hundred and thirty-five (435) ml of water and 5 ml of concentrated sulfuric acid were added to the flask providing a total volume of 450 ml in the distillation flask. Heating mantles controlled by variable voltage transformers were used to adjust the distillation apparatus (1) to provide a distillation rate of 1,200 ml per 90 ± 5 min and (2) to maintain the volume in the distillation flask at 450 ± 10 ml throughout the 90-min distillation period.

After distillation, the condenser was washed several times with water, and the washings were added to the contents of the graduated cylinder. The 1,200+ ml of distillate was quantitatively transferred to a 1,500-ml beaker and quantitatively neutralized to a phenophthalein end point with $N/100$ sodium hydroxide. Ten replicate 10-ml aliquots of each acid were distilled using the above procedure.

Acid extraction and preparation of samples for analysis

The frozen egg samples were thawed and a 200-g aliquot was accurately weighed into a tared 1,000-ml wide-mouth Erlenmeyer flask. Five hundred milliliters of water were added, followed by 75 ml of $N/1$ sulfuric acid. The contents of the flask were gently swirled until thoroughly mixed. Seventy-five milliliters of 20% phosphotungstic acid solution were added, and the contents of the flask made to 1,000 g with water. The flask was stoppered and vigorously shaken for 1 min. The sample was divided and filtered through two 24-cm folded filter papers (Whatman No. 12) into 600-ml beakers. Filtration proceeded for 60 min. The filtrates were then combined and 450 ml of the filtrate were transferred by pipette to the distillation flask of the steam distillation assembly, and the acids were distilled and collected using the procedure outlined above.

The distillate was quantitatively neutralized with $N/100$ sodium hydroxide using phenolphthalein as the indicator. An excess of base (usually 1–1.5 ml of $N/10$ sodium hydroxide) was added to the neutralized distillate and the volume was reduced using a hot plate and steam bath. More base was added when the color became discharged. When the volume reached approximately 25 ml, the distillate was transferred to a 50-ml beaker to approximately 1 to 2 ml on a steam bath.

The sodium salts of the acids were quantitatively transferred to a 12-ml graduated centrifuge tube, using a 1:1 (v/v) mixture of acetone and water. The acetone and water were evaporated under a stream of dry air on a steam bath. The dried salts were loosened with a micro-spatula and 1 ml of the dichloroacetic mixture was added to the centrifuge tube.

The contents of the tube were mixed for 1 min on a Vortex Jr. mixing apparatus to insure complete dispersal of the salts and liberation of the volatile acids. A volume of the butyl octanoate internal standard (as specified for the acid content of the sample in Table 3) was pipetted into the tube. The sample was diluted to 2 ml, stoppered and mixed again with the Vortex mixer. The precipitated sodium dichloroacetate was allowed to settle out and one μl of the clear solution was injected into the chromatograph.

Interpretation of data

The peak heights of acetic, propionic and butyric acids and butyl octanoate were measured from the chromatograms of the samples. Rearrangement of the R value equation gave the following formula:

$$c_A = (h_A)\,(c_{IS})\,/(R_A)\ (h_{IS})$$

Since R values for each acid were previously determined, unknown acid concentrations were calculated by substituting the appropriate peak heights and R values in the above equation. After determining the concentration of acetic, propionic and butyric acids present in the injected aliquot, the concentration value for acetic, propionic and butyric acids was multiplied by 5355, 4631 and 4444, respectively. These factors converted the quantity of acetic, propionic and

Table 2—Operating parameters for gas-liquid chromatograph.

Controlled factor	Operating condition: Acetic, propionic & butyric mixtures	Operating condition: Formic and acetic mixture
Column	72 in. × 1/4 in. copper tubing	Same
Carrier gas	Helium	Same
Pressure	40 psi	Same
Flow rates	A column—50 ml/min	76 ml/min
	B column—51 ml/min	78 ml/min
Hydrogen	20 psi	Same
Air	640 ml/min @ 35 psi	Same
Temperatures	Detector 250°C	Same
	Injection port 250°C	Same
	Column 120°C	60°C
Chart speed	30 in./hr	Same
Sample size	1 μl	Same
Range	10	Same
Attenuation	4–32	Same

Table 3—Volume of internal standard added/ml of base required to neutralize steam distillate.

N/100 NaOH for neutralization of steam distillate (before addition of excess) (ml)	Internal standard added (concentration)		Total volume after dilution (ml)
1.0–10.0	0.10	1:10 dilution of stock	2.0
10.1–20.0	0.25	1:10 " " "	2.0
20.1–30.0	0.50	1:10 " " "	2.0
30.1–50.0	0.10	Stock solution	2.0

butyric acids present in the injected aliquot to mg of acetic, propionic and butyric acids/100 g of egg. These conversion factors were derived as follows:

	Column a	Column b	Column c	Column d
Acetic	$\left[\frac{2000}{1}\right]$	$\left[\frac{1000}{450}\right]$	$\left[\frac{100}{90.4}\right]$	$\left[\frac{100}{200}\right]$
Propionic	$\left[\frac{2000}{1}\right]$	$\left[\frac{1000}{450}\right]$	$\left[\frac{100}{96.8}\right]$	$\left[\frac{100}{200}\right]$
Butyric	$\left[\frac{2000}{1}\right]$	$\left[\frac{1000}{450}\right]$	$\left[\frac{100}{100}\right]$	$\left[\frac{100}{200}\right]$

where:

Column a is the dilution factor when a one μl (0.001 ml) aliquot for each injection was taken from a two ml sample.

Column b is a factor which corrects for the fact that only 450 ml (g) of the original 1,000 g of egg-water-phosphotungstic acid-sulfuric acid mixture was used.

Column c is a factor which accounts for the quantity of acetic, propionic and butyric acids recovered by steam distillation.

Column d is a factor which converts mg acid in 200 g egg to mg acid in 100 g egg.

Calibration of chromatograph (formic and acetic acids)*NN*

Approximately 1/2 g of each of butyl formate and butyl acetate (K & K Labs) were accurately weighed into the same 10-ml volumetric flask, made to volume with acetone and designated as the stock solution.

Two dilutions, 1:10 and 1:50, of the stock solution were prepared and designated as "dilute stock solution A" and "dilute stock solution B," respectively. Various volumes of the diluted stock solutions of the standard butyl esters were injected into the chromatograph to provide a range of concentration for each butyl ester at a given sensitivity. Triplicate injections at each sensitivity were made and the peak heights corresponding to each concentration at a given sensitivity were measured. The peak heights and concentrations were used in the calculation of the acid concentrations in the unknowns.

Butyl esterification efficiency for formic and acetic acids

Ten milliliter aliquots of accurately standardized *N*/20 formic and acetic acid solutions were pipetted into a 50-ml standard taper round-bottom flask. The water was evaporated on a steam bath under a stream of dry air. One milliliter of 1-butanol, two drops of concentrated hydrochloric acid and approximately 500 mg of anhydrous sodium sulfate were added to the flask containing the dried material. The flask was connected to a condenser equipped with a drying tube filled with soda lime and the mixture was gently refluxed for 2 hr with a 50-ml heating mantle connected to a variable voltage transformer set at 40.

After esterification, the contents of the flask were quantitatively transferred to a 5-ml volumetric flask and made to volume with acetone. One-μl aliquots were injected into the chromatograph and the formic and acetic acids were recovered as butyl formate and butyl acetate.

The following formula was applied to calculate the concentration of the butyl formate and butyl acetate recovered by using the peak heights of the concentrations at a given sensitivity of the standards chromatographed as described above:

$$\text{Concentration of unknown butyl ester} = \frac{(\text{Peak height of unknown ester}) \times (\text{Concentration of standard butyl ester})}{\text{Peak height of standard butyl ester}}$$

Table 4—Determination of esterification efficiency of formic and acetic acids.

Amount in 10 ml aliquot (mg)	Equivalent as butyl formate in 10 ml aliquot (mg)	Recovery from GLC l/(mg)	Average recovery (%)[1]
Formic acid			
20.8	46.6	49.30 ± 0.04	106
Acetic acid			
20.9	40.3	38.35 ± 0.10	95

[1] Average of ten replications ± standard deviation of the mean.

Acid extraction and preparation for analysis

The formic and acetic acids were extracted from the frozen whole egg samples as outlined for acetic, propionic and butyric acids.

The steam distillate was reduced to approximately 10 ml in volume on a hot plate and steam bath and quantitatively transferred to a 50-ml round-bottom flask. The distillate was evaporated to dryness on a steam bath under a stream of dry air.

The dried sodium salts of formic and acetic acid were converted to their butyl esters using the procedures outlined above, beginning with "one ml of 1-butanol . . ." After butylation was complete, the sides and joint of the condenser were rinsed with 1 ml of acetone and the washings added to the contents of the round bottom flask. The contents of the flask were quantitatively transferred to a 5-ml volumetric flask through a micro-funnel. The round-bottom flask was rinsed 5 times with 1/2-ml portions of acetone, and the washings added to the contents of the volumetric flask. The contents of the flask were made to volume with acetone and 1-μl aliquots were injected into the chromatograph.

Interpretation of data

From the chromatograms of the samples, the peak heights of butyl formate and butyl acetate were measured. The mg concentration of butyl formate and butyl acetate re-

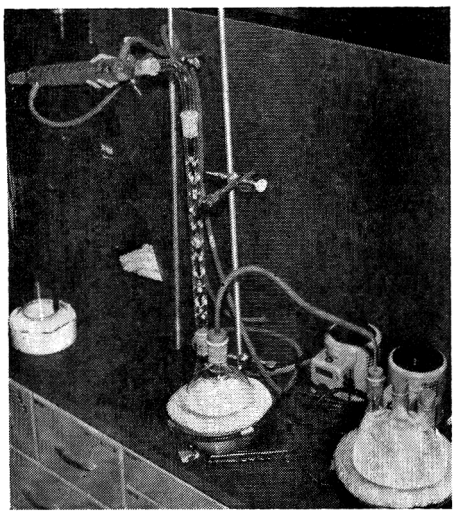

Fig. 1—Photograph of steam distillation assembly.

covered was calculated, using the formula previously given.

After determining the mg concentration of butyl formate and butyl acetate present in the injected aliquot, the concentration value for butyl formate and butyl acetate was multiplied by 3205 and 3575, respectively. These factors convert the quantity of butyl formate and butyl acetate present in the injected aliquot to mg of formic and acetic acids/100 g of egg. The formulas for determining these conversion factors were derived as follows:

where:

Column a is a factor which converts butyl formate and butyl acetate to formic and acetic acid.

Column b is the dilution factor when one μl (0.001 ml) aliquot for each injection was taken from a 5-ml sample.

Column c is a factor which corrects for the fact that only 450 ml (g) of the original 1,000 g of egg-water-phosphotungstic-sulfuric acid mixture was used.

Column d is a factor which accounts for the 106 and 95% efficiency of butylation for formic and acetic acids, respectively.

Column e is a factor which accounts for the 77.4 and 90.4% acid recovery from steam distillation for formic and acetic acids, respectively.

Column f is a factor which converts mg acid/200 g egg to mg acid/100 g egg.

RESULTS & DISCUSSION

Development of acceptable procedures

Four different types of liquid phases of various percentages were evaluated in this study. The liquid phases used were Carbowax 1540 at 10, 15 and 25% levels; Carbowax 20M at 10, 15 and 20% levels; Apiezon L at 5, 7.5 and 10% levels; and ethylene glycol adipate (EGA) at 15 and 20% levels. Commercially prepared standards of the butyl esters and acids were chromatographed on these columns under various temperature conditions and carrier gas flow rates and the columns were evaluated for their effect on peak symmetry and resolution of the standard esters and acids.

The two Carbowaxes gave good separation of the C_2–C_4 acids; however, the peaks of the acids and the butyl esters on these columns and the Apiezon L columns were not symmetrical and poor resolution between the solvent and butyl formate occurred on the Apiezon L columns. Except for differences in retention times, necessitating changes in the temperature conditions of the experimental runs, the ethylene glycol adipate at either the 15 or 20% level was quite satisfactory in regard to peak symmetry and resolution for the C_2–C_4 acids. At the 20% level, however, a slightly greater resolving power between the butyl acetate and butanol were observed, hence the 20% EGA column was adopted for use in these studies.

The quantitative determination of formic acid, per se, present in eggs could not be accomplished using the chromatograph available since formic acid readily decomposed to CO_2 and water on contact with metal. In an attempt to overcome this problem, an all-glass column and injection system was installed in the chromatograph. However, no response for formic acid at any sensitivity could be detected. It was theorized that the formic acid decomposed on contact with the metal of the detectors after leaving the glass column and prior to reaching the flame. Since the use of butyl derivatives for lactic and succinic acids had proven successful (Steinhauer et al., 1969) butyl derivatives of formic and acetic acids were prepared and chromatographed.

Acid esterification

The acid esterification yields, shown in Table 4, indicated that formic and acetic acids could be quantitatively recovered. However, due to short retention times of butyl formate, butyl acetate and the excess butanol, no suitable internal standard could be used without an overlapping of the peaks and incomplete resolution. Higher percentage liquid phases (up to 30%) and longer columns (up to 10 ft) did not sufficiently improve resolution to allow the use of an internal standard. Therefore, quantitation was achieved by injecting known concentrations of commercially available butyl formate and butyl acetate.

The peak heights of the standards were compared with the peak heights of the unknowns, and the concentrations of the unknowns were calculated. The elution positions of butyl formate, butyl acetate and butanol are shown in Figure 2. Butyl acetate was esterified with good precision (92–97%) as was butyl formate (105–107%); however, the reason for the 105% recovery of butyl formate could not be ascertained.

	Column					
	a	b	c	d	e	f
Formic	[0.447]	$\left[\frac{5000}{1}\right]$	$\left[\frac{1000}{450}\right]$	$\left[\frac{100}{106}\right]$	$\left[\frac{100}{77.4}\right]$	$\left[\frac{100}{200}\right]$
Acetic	[0.517]	$\left[\frac{5000}{1}\right]$	$\left[\frac{1000}{450}\right]$	$\left[\frac{100}{95}\right]$	$\left[\frac{100}{90.4}\right]$	$\left[\frac{100}{200}\right]$

Table 5—Response values, R, of acetic, propionic and butyric acids relative to butyl octanoate.

Range of acid concentration in sample (mg)	Chromatography		R value			
	Range	Attenuation	Low	High	Range	Average
			Acetic Acid			
0.10–0.50	10	4	0.660	0.758	0.098	0.720
0.51–1.00	10	8	0.716	0.765	0.049	0.739
1.01–2.50	10	16	0.571	0.642	0.071	0.609
2.51–7.50	10	32	0.705	0.757	0.052	0.733
			Propionic acid			
0.10–0.50	10	4	0.764	0.858	0.094	0.825
0.51–1.00	10	8	0.860	0.910	0.070	0.888
1.01–2.50	10	16	0.635	0.742	0.107	0.685
2.51–7.50	10	32	0.838	0.891	0.053	0.864
			Butyric acid			
0.10–0.50	10	4	0.600	0.672	0.072	0.645
0.51–1.00	10	8	0.686	0.728	0.042	0.708
1.01–2.50	10	16	0.558	0.643	0.085	0.597
2.51–7.50	10	32	0.711	0.751	0.040	0.730

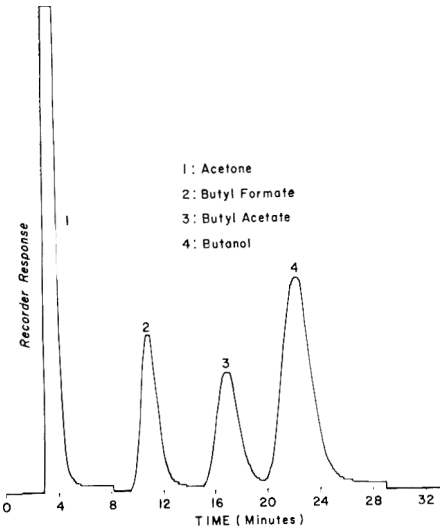

Fig. 2—A gas chromatogram of butyl formate and butyl acetate showing their order of elution and retention times.

Internal standard

The elution position of the internal standard, butyl octanoate, relative to the C_2–C_4 acid, is shown in Figure 3. In this laboratory, methyl enanthate as the internal standard for the C_2–C_4 acids as described by Shelley et al. (1963) was unsuccessful, since its retention time was the same as that of acetic acid. Butyl octanoate was selected because its retention time was greater, yet sufficiently close to the C_2–C_4 acids to permit rapid analysis.

The response values of the C_2–C_4 acids relative to butyl octanoate were calculated according to the procedure previously described by Shelley et al. (1963). The R values over a wide range of acid concentrations relative to the internal standards are presented in Table 5. Shelley et al. (1963) reported that variations in R values decreased as the concentration of the internal standards and the compounds under study increased. As shown in Table 5, the R value variations generally decreased with increasing concentration of internal standards, acids and butyl esters.

Table 6—Recovery of formic, acetic, propionic and butyric acids by steam distillation.

Acid	ml *N*/100 NaOH required for 10 ml aliquot[1]	ml *N*/100 NaOH required for recovered acid[2]	Average percent recovery
Formic	38.92	30.10 ± 2.31	77.4
Acetic	45.40	41.04 ± 2.29	90.4
Propionic	38.30	37.09 ± 2.29	96.8
Butyric	36.25	36.28 ± 0.04	100.0

[1] Average of three replications.
[2] Average of ten replications ± standard deviation of the mean.

Analysis of C_2–C_4 acids

A modified procedure of Shelley et al. (1963) was adopted for the quantitative analysis of the short-chain acids. These authors used an exact amount of dichloroacetic acid to acidify the sodium salts of the acids and to improve the stability of the final solution prior to GLC analysis. In this study, an excess of dichloroacetic acid was used to release the acids from their sodium component with no apparent loss of solution stability or changes in retention times. The excess dichloroacetic acid had the same retention time as acetone, and consequently did not interfere with the resolution of the acids.

The use of phosphoric acid on the columns was also omitted in this study since the material tended to bleed off the column during prolonged runs. This caused fouling of the detector and resulted in recorder noise and pen spiking.

The steam distillation apparatus used in these studies (see Fig. 1) was a modification of the apparatus used in standard AOAC (1960) determinations. Recoveries averaged 77.4% for formic acid; 90.4% for acetic acid; 96.8% for propionic acid; and 100% for butyric acid (Table 6).

The use of larger distillation flasks and steam generators as described under Experimental Procedures improved the precision of the rate of distillation since the larger volumes were not affected by temperature and air movement fluctuations as much as the standard AOAC apparatus. The Vigreux fractionating column was inserted between the distillation flask and the condenser to prevent entrainment of the sulfuric acid and the other compounds which would affect the quantitative neutralization of the distillate.

At the initiation of these studies, an attempt was made to extract all the C_1–C_4 acids, and lactic and succinic acids using the liquid-liquid extraction procedure, as suggested by Ramsey (1963). The acids were trapped in 25 ml of *N*/10 KOH solution in the side-arm flask of the extractor. Following evaporation to dryness, the potassium salts of the acids were esterified with butanol, made to volume and chromatographed using temperature programming. Butyl formate, butyl acetate and dibutyl succinate were successfully recovered by this procedure, but the excess butanol, butyl propionate, butyl butyrate and butyl lactate exhibited identical retention times.

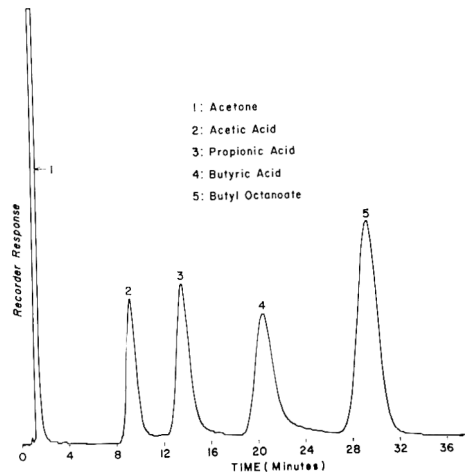

Fig. 3—A gas chromatogram of acetic, propionic and butyric acid and butyl octanoate showing their order of elution and retention times.

Recovery of acids

A comparison of the recoveries of acetic, propionic and butyric acids using

Table 7—Recovery of acetic, propionic and butyric acids from frozen whole eggs using GLC and AOAC (1960) procedures.

Sample	mg/100 g egg (added)	mg/100 g egg (recovered) GLC[1]	mg/100 g egg (recovered) AOAC[2]	Average percent recovery GLC	Average percent recovery AOAC
		Acetic			
1	1.17	1.33 ± 0.05	Trace	114	—
2	3.17	3.13 ± 0.13	3.53 ± 0.07	99	113
3	6.34	6.45 ± 0.06	6.20 ± 0.08	102	98
4	12.67	12.70 ± 0.08	12.77 ± 0.07	100	101
		Propionic			
1	1.38	1.38 ± 0.03	Trace	100	—
2	3.72	3.55 ± 0.38	3.73 ± 0.12	98	100
3	7.44	7.43 ± 0.03	7.43 ± 0.21	100	100
4	14.87	14.80 ± 0.14	14.87 ± 0.16	101	100
		Butyric			
1	1.70	1.75 ± 0.13	Trace	103	—
2	4.59	4.70 ± 0.18	4.43 ± 0.16	102	97
3	9.29	9.08 ± 0.15	9.35 ± 0.24	98	101
4	18.37	18.18 ± 0.03	18.23 ± 0.07	99	99

[1] Average of four replications ± standard deviation of the mean.
[2] Average of three replications ± standard deviation of the mean.

Table 8—Recovery of formic and acetic acids from frozen whole eggs using GLC and AOAC (1960) procedures.

Sample	mg/100 g egg (added)	mg/100 g egg (recovered)		Average percent recovery	
		GLC[1]	AOAC[2]	GLC	AOAC
		Formic			
1	1.25	1.30 ± 0.07	1.33 ± 0.05	104	106
2	2.47	2.68 ± 0.07	2.48 ± 0.06	109	100
3	4.94	5.01 ± 0.04	4.91 ± 0.05	101	99
4	9.88	9.72 ± 0.07	9.93 ± 0.01	98	101
5	24.76	23.68 ± 0.11	23.64 ± 0.07	96	95
		Acetic			
1	1.15	1.17 ± 0.10	Trace	102	
2	3.60	3.15 ± 0.08	3.34 ± 0.00	95	101
3	6.60	6.61 ± 0.10	6.45 ± 0.05	100	98
4	13.20	13.13 ± 0.10	13.03 ± 0.17	99	99
5	33.32	32.75 ± 0.57	32.98 ± 0.02	98	99

[1] Average of four replications ± standard deviation of the mean.
[2] Average of three replications ± standard deviation of the mean.

GLC and AOAC is presented in Table 7, and the results show that the standard deviation of the mean for both procedures is similar. Again, the accuracy of both methods improves with increasing concentrations of acids. Here, the main advantage of the GLC procedure is the detection of lower quantities of the C_2–C_4 acids as compared to the standard AOAC procedure.

The recoveries of formic and acetic acids by GLC and AOAC procedures (as their butyl derivatives) are presented in Table 8. Considering that no internal standard was employed, the precision of the GLC method, as shown by the relatively small standard deviations of the means, compared favorably with the precision of the AOAC methods, and there were no significant differences in accuracy between the two methods.

The wholesomeness of frozen egg products is presently evaluated by a combination of bacterial, chemical and sensory methods. Hillig et al. (1942) reported that the presence of decomposition products in frozen eggs was confirmed when the eggs contained a direct microscopic count of over 5 million cells/g of egg with determinable amounts of formic or acetic acid, or lactic acid in excess of 7 mg/100 g egg. Succinic acid was offered as an additional chemical index of decomposition. Since the chemical data to support these conclusions were variable, more accurate analytical methods or methods for determination of other decomposition products seems desirable. The use of GLC procedures reported here, plus those reported for lactic and succinic acids (Steinhauer et al., 1969) offer new procedures for accurate determination of frozen eggs. These procedures should now be evaluated by analyzing a large number of commercial egg samples obtained from processors varying in output and source of eggs.

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The Anthocyanins of Strawberry, Rhubarb, Radish and Onion

SUMMARY—The presence of a large number of previously unnoticed anthocyanins was demonstrated in strawberry, rhubarb, radish and onion. The number of anthocyanins found in this survey and those reported previously (in brackets) in each plant were: strawberry: 6 (4), rhubarb: 3 (2), radish: 13 (5), onion: 8 (3). Some of the chromatograms indicated that the number of anthocyanins present was even greater than that. A quantitative difference was found between the anthocyanin pattern of the examined varieties. The survey was carried out using paper chromatography on Whatman No. 3 MM paper with two new solvent systems of high resolving power. The solvents were: 1-butanol-benzene-formic acid-water (100:19:10:25) and 1-butanol-formic acid-water (100:25:60).

INTRODUCTION

COLOR is an important attribute of most fruits and vegetables as well as their products; the identification of the compounds responsible for color is of interest. The red and blue colors of plants are due to the presence of anthocyanin pigments. The deterioration of red color is connected with the anthocyanin composition of the plant material (Daravingas et al., 1965; Robinson et al., 1966; Starr et al., 1968) therefore a better knowledge of these pigments is an aid in solving the discoloration problem. The adulteration of fruit and vegetable products can be detected by the chromatographic pattern of anthocyanins as demonstrated with wine (Ribéreau-Gayon, 1959); grape juice (Fitelson, 1967; Mattick et al., 1967) and other fruit juices (Jorysch et al., 1966; Fitelson, 1968).

The anthocyanins in several fruits and vegetables have been identified in the last decade (Harborne, 1967a). The anthocyanin composition of only a few fruits and vegetables is known completely. The anthocyanins occurring only in minute quantities in any given fruit or vegetable are little known. Previous work (Fuleki et al., 1967a) showed that the number of anthocyanins present in the American cranberry is greater than previously thought. This led to the present chromatographic survey of the anthocyanin composition of some readily available fruits and vegetables with the aid of two new solvent systems of high resolving power.

The anthocyanins reported to be present in the plant material included in this survey are listed in Table 1. As indicated in the table the authors employed identification methods of varying degree of accuracy. These methods can be classified as follows:

Classical method

This method, perfected by Robinson et al. (1931, 1932), consists of a series of color and distribution tests on the par-

Table 1—The anthocyanins identified in strawberry, rhubarb, radish and onion.

Plant material	Variety	Identification method[1]	Pigment[2]	Reference
Strawberry (*Fragaria x ananassa* Duch.),[3] fruit	—	I	Pl 3-Gl	Robinson et al. (1932)
	—	I	Pl 3-monoside	Lawrence et al. (1939)
	Bailey	I	Pl 3-Gl	Sondheimer et al. (1948)
	—		Pl 3-Gl Pl diglycoside	Akuta et al. (1954)
	—	II	Pl 3-Gl	Robinson et al. (1955)
	Shasta, Marshall Culver	III	Cy 3-Gl	Lukton et al. (1955)
		III	*Pl 3-Gl*, Cy 3-Gl, Pl glycoside	Sondheimer et al. (1956)
	—	II	Cy and *Pl* monoside	Fouassin (1956)
	Sovereign	II	Pl, Cy and an unidentified third glycoside	Lamort (1958)
	—	II	Cy and Pl glycoside	Nybom (1964)
	—	II	*Pl 3-Gl*, Cy 3-Gl	Blundstone et al. (1966)
Rhubarb (*Rheum rhaponticum* L.), petiole	Sutton's varieties	I	Cy 3-bioside	Robinson et al. (1931)
		III	Cy 3-Gl, Cy 3-Ru	Gallop (1965)
	—	III	Cy 3-Gl, Cy 3-Ru	Blundstone et al. (1966)
Radish (*Raphanus sativus* L.), root	—	II	five acylated Pl glycoside	Fouassin (1956)
	Scarlet Globe	III	Pl 5-Gl, 3-diGl (Coum) or (Fer)	Harborne et al. (1957)
	Iwakumi-aka	III	Pl 5-Gl, 3-diGl (Coum) or (Fer + Caf)	Ishikura et al. (1962)
	purple var.	III	Cy 5-Gl, 3-Sop (Coum) or (Fer)	Harborne (1963)
	red var.	III	Pl 5-Gl, 3-Sop (Coum) or (Fer)	Harborne (1963)
	Iwakumi-aka	III	Pl 5-Gl, 3-diGl (Coum) or (Fer) or (Caf) or (Coum + Fer) or (Coum + Fer + Caf)	Ishikura et al. (1963)
	—	II	Pl	Nybom (1964)
Red onion (*Allium cepa* L.), skin	Sutton's Blood Red	I	Cy 3-pentoseglycoside[4]	Robinson et al. (1932)
	—	II	Cy monoside, diglycoside and a third Cy derivative	Fouassin (1956)
	Southport Red Globe	II	Pn 3-Ar	Brandwein (1965)

[1] I = Classical, II = Simple chromatographic, III = Chromatographic and spectroscopic, IV = Degradative method. Detailed explanation of these terms are given in the text.

[2] Pl = pelargonidin, Cy = cyanidin, Gl = glucoside, Ar = arabinoside, Ru = rutinoside, Sop = sophoroside, Coum = p-coumaric acid, Fer = ferrulic acid, Caf = caffeic acid. The acyl group is in brackets. The major pigment is in italics when this is indicated in the literature.

[3] This includes the cultivated varieties identified as *F. virginiana* by the authors.

[4] The authors identified the pigment as cyanidin 3-pentosegycoside and indicated that most pentoseglycoside is a rutinoside.

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tially purified pigment extract. Since the individual anthocyanins are not separated, only the most abundant anthocyanin can be identified. The accuracy of this method is rather limited.

Simple chromatographic method

The advance of chromatographic techniques permitted the preparation of pure individual anthocyanins and the cumbersome distribution test was replaced by R_f value determinations. Identification is based on R_f values, absorption maxima and color reactions obtained with the anthocyanin and/or anthocyanidin. Methods with varying degree of accuracy are included in this group, all of them being characterized by the fact that chromatographic techniques are used but the evidence provided is insufficient for a complete identification. The method is only reliable for the identification of the anthocyanin class in which the particular pigment belongs.

Chromatographic and spectroscopic method

This method is based mainly on Bate-Smith and Harborne's original work and described in Harborne's recent book (1967b). The identification is based on R_f and spectral values obtained with the highly purified individual anthocyanin and its degradation products produced by acid or enzymic hydrolysis. The method includes a saponification test and identification of the acid component when acylation is suspected. This method is satisfactory for reliable identification of the anthocyanin.

Degradative method

When dealing with rare anthocyanidins the above outlined method might not be sufficient. To be certain of the identification, additional data are obtained on the aglycon by analyzing the fragments produced by the chemical degradation of the anthocyanidin.

A close examination of the data (Table 1) shows that there is disagreement among the authors, not only concerning the nature of the pigments, but also on the number of anthocyanins present in the plant material.

EXPERIMENTAL

Plant materials

The anthocyanin rich tissues of the plants were used as pigment sources. The plant materials used were grown on the experimental plots of the Kentville Research Station.

Strawberries (*Fragaria* x *ananassa* Duch.). Fully ripened berries of the following varieties were used: Acadia, Gorella, K-60-98, K-63-280, Midway, NJ-857, Redcoat, Senga Sengana and Sparkle. The center part of the berries was discarded when found to contain only a low concentration of anthocyanin. To remove the bulk of the sugar the juice was squeezed out of the berries and only the pomace was used as source for the anthocyanins.

Rhubarb (*Rheum raponticum* L.). The "forced" petioles of the German Wine, Sunrise, Sutton's Seedless and Valentine varieties were used. The pigments were extracted from the anthocyanin rich base and the peeled epidermal layer of the petioles.

Radish (*Raphanus sativus* L.). The roots of the French Breakfast and Scarlet White Tip varieties were supplied by local growers. Two unidentified early red varieties grown in Ohio and Texas were obtained from the market. The pigments were extracted from the scraped epidermal layer.

Onion (*Allium cepa* L.). The bulbs of the Ruby and an unidentified Texas grown purple onion variety were used. The outer dry scale and the anthocyanin containing part of the fleshy scale-leaves were removed and extracted separately.

Extraction

The anthocyanin containing tissue was soaked in diethylether for 4–5 hr to facilitate the subsequent extraction of anthocyanins and remove some of the impurities which are usually extracted with methanol. The ether extract was discarded and the plant material was covered with the extracting solvent and leached overnight at room temperature. To improve the extraction of the diglycosides and triglycosides, 1% HCl in methanol was alternated with 1.5*N* HCl-methanol (15:85) as extracting solvent. The extraction was repeated 3–4 times as required. The volume of the more concentrated extract was reduced by evaporation under atmospheric pressure. The dilute extracts were concentrated *in vacuo* in all glass evaporator at 25°C. The filtered combined concentrated extracts were used for chromatographic analysis.

Chromatography

The extract was applied to 46 x 57 cm sheets of Whatman No. 3 MM paper as 10 to 17 cm streaks with a 0.2-ml long tip pipet. Descending chromatography was carried out either in a Model A-300 Chromatocab (Research Equipment Corp., Oakland, Calif.) or in a Panglass Chromatank Model 500 (Shandon Sci. Co. Inc., Sewickley, Pa.) all glass chromatography tank. The following developing solvents were used:

BBFW 1-butanol-benzene-formic acid-water (100:19:10:25), aged three days, upper phase (Fuleki et al., 1967b).

BFW 1-butanol-formic acid-water (100:25:60), upper phase.

To achieve maximum separation the development was continued until the fastest moving pigment reached the area within 2–3 cm from the bottom edge of the paper.

Evaluation of the chromatograms.

The chromatograms were examined under a tungsten light source and over a Black Ray Model C-50 long wave and Mineralight Model C-51 short wave ultraviolet transilluminators (Ultra-Violet Products Inc., San Gabriel, Calif.). The point of maximum concentration for each band was marked over the Mineralight. The numbering of the pigment bands began with the fastest moving pigment. The colors of the bands and the changes produced by treating the chromatograms with chromogenic reagents were also noted. The following reagents were used for this purpose:

$AlCl_3$ 3 g crystalline aluminum chloride dissolved in 100 ml 75% ethanol.

PbAc 1 g neutral lead acetate dissolved in 100 ml 75% ethanol (Fuleki et al., 1967c).

The relative quantities reported in Table 2 were estimated visually. A more accurate measurement was carried out in one case with the rhubarb varieties. The rhubarb anthocyanins were eluted with 1% HCl in methanol from the chromatograms and their quantities were measured at the absorption maxima with a spectrophotometer. Since the extinction values of the pigments involved were not all known, the possible differences were disregarded in the calculations.

Calculation of the relative mobility of the pigments

Since the solvent front overran the paper, the mobility of the pigment bands was calculated instead of the customary R_f values. Since cyanidin 3-glucoside was known to be present in strawberry and rhubarb, and the No. 4 onion and No. 5 radish pigments had similar mobility to that of cyanidin 3-glucoside, these pigment bands were selected as bases of comparison. The mobility of each pigment relative to cyanidin 3-glucoside or to the No. 4 onion and No. 5 radish pigments (R_{cy} in the following) was calculated for each chromatogram using the following equation:

$$R_{cy} = \frac{\text{distance traveled by pigment "X"} \times 100}{\text{distance traveled by cyanidin 3-glycoside or equivalent}}$$

The av R_{cy} values reported in Table 2 for each pigment were calculated from the R_{cy} values obtained on several chromatograms. The number of measurements on which the reported R_{cy} values were based are also given in Table 2. There are cases where the number of measurements are not the same for each pigment of the same plant. This is due to the fact that in some cases the fastest moving pigment band(s) overran the paper. In other cases the concentration of some pigments was below the threshold of detection or overlapping of the pigment bands occurred.

RESULTS & DISCUSSION

THE RESULTS presented in Table 2 show that a number of previously unidentified anthocyanins were found in the edible parts of strawberry, rhubarb, radish and onion. Some of these pigments occurred in fairly large quantities. In the following, the pigment pattern reported in the literature (Table 1) is compared with that found in this survey (Table 2).

Strawberry

Two "new" anthocyanins were found

in addition to the two identified and the two previously noted but unidentified pigments (Table 2). The presence of pelargonidin and cyanidin 3-glucoside is well established and these were the most abundant pigments (No. 2 and 4 respectively) in all the varieties examined. Akuta et al. (1954) found in addition to the pelargonidin 3-glucoside a pelargonidin diglycoside in strawberries.

The presence of a fourth pigment which was less hydrophilic than pelargonidin 3-glucoside, as indicated by its chromatographic mobility, was demonstrated by Sondheimer et al. (1956) and Lamort (1958). The first named authors indicated that this pigment is a pelargonidin derivative. The anthocyanin band numbered 1 on our chromatograms appears to be identical with this pigment. In addition to the four anthocyanins mentioned above two new pigments were found (Table 2). An additional orange-brown pigment band with R_{Cy} values of 25 and 36 in BBFW and BFW respectively appeared on most chromatograms, in very low concentration. Judged by its color this pigment may also be a pelargonidin glycoside. A quantitative rather than qualitative difference was found in the anthocyanin composition of the strawberry varieties examined (Table 3).

Table 2—A survey of the anthocyanins separated by paper chromatography.

Pigment band		Quantity[1]	Color[2] Untreated	$AlCl_3$	PbAc	R_{Cy}[3] BBFW	BFW	Tentative identification[4]
Strawberry,	1	+	SR	SR	GR	211(8)	190(9)	Pl monoside
	2	++++	SR	SR	GR	165(8)	159(10)	Pl 3-Gl
	3	+	SR	SR	GR	118(8)	120(10)	Pl monoside[5]
	4	++	R	V	B	100(8)	100(10)	Cy 3-Gl
	5	+	SR	SR	GR	81(8)	80(10)	Pl bioside
	6	<+	SR	SR	GR	50(8)	54(9)	Pl trioside[5]
Rhubarb,	1	++++	R	V	B	100(55)	100(3)	Cy 3-Gl
	2	++++	R	V	B	79(55)	82(3)	Cy 3-Ru
	3	+	R	V	B	59(55)	62(3)	Cy bioside[5]
Red Radish,	1	+	SR	SR	GR	149(11)	170(10)	Pl 5-Gl,3-Sop(Coum + Fer + Caf)
	2	++	SR	SR	GR	136(11)	147(11)	Pl 5-Gl,3-Sop (Coum + Fer)
	3	+	SR	SR	GR	126(11)	134(11)	Pl glycoside[6]
	4	++++	SR	SR	GR	112(9)	116(9)	Pl 5-Gl,3-Sop (Coum)
	5	++++	SR	SR	GR	100(14)	100(14)	Pl 5-Gl,3-Sop (Fer)
	6	++++	SR	SR	GR	85(14)	87(13)	Pl 5-Gl,3-Sop (Caf)
	7	+++	SR	SR	GR	71(14)	77(14)	Pl glycoside[5]
	8	<+	SR	SR	GR	64(7)	66(5)	Pl glycoside[5]
	9	++	SR*	SR	GR	51(14)	63(12)	Pl glycoside[5]
	10	+	SR*	SR	GR	39(14)	47(12)	Pl glycoside[5]
	11	+++	SR*	SR	GR	27(14)	37(12)	Pl 5-Gl,3-Sop[5]
	12	+	SR*	SR	GR	22(3)	30(11)	Pl glycoside[6]
	13	+	SR*	SR	GR	10(5)	14(12)	Pl glycoside[5]
Red Onion,	1	+	R	V	B	171(17)	169(10)	Cy monoside[5]
	2	++	R	V	B	145(43)	132(28)	Cy monoside[6]
	3	+	R	R	GR	121(53)	118(29)	Pn monoside[5]
	4	++++	R	V	B	100(61)	100(31)	Cy monoside
	5	+	R	V	B	78(13)	70(1)	Cy bioside[5]
	6	++	R	V	B	50(61)	66(28)	Cy bioside
	7	++	R	V	B	30(60)	40(31)	Cy bioside
	8	+	R	V	B	21(44)	26(19)	Cy diglycoside[5]

[1] The relative quantity of the pigments was estimated visually and indicated as follows: ++++ = major, +++ = intermediate, ++ = minor, + = trace quantity.
[2] Color code: B = blue, G = gray, R = red, S = scarlet, V = violet, * = fluorescent yellow under UV light.
[3] R_{Cy} = chromatographic mobility relative to Cy 3-Gl or to the No. 5 radish or No. 4 onion pigments. The number of measurements on which the av R_{Cy} values were based are given in brackets.
[4] The abbreviations used were the same as in Table 1.
[5] Previously undetected Acys.

Rhubarb

Three anthocyanin pigments were found in rhubarb (Table 2). Two of these pigments were identified previously but this is the first report on the occurrence of a third pigment. Gallop (1965) identified the two major pigments in rhubarb as cyanidin 3-glucoside and cyanidin 3-rutinoside. The No. 1 and 2 pigment bands in Table 2 corresponded to these pigments. The third anthocyanin was present in small quantities in all the examined varieties. Table 4 shows the approximate quantities of each pigment in the varieties included in this survey.

A faint diffuse anthocyanin band was observed in front of the No. 1 pigment on some of the chromatograms. This fourth anthocyanin had an R_{Cy} value of 126 and 123 in BBFW and BFW respectively. The low R_{Cy} values excluded the possibility that this pigment was an anthocyanidin. In addition to the anthocyanins two reddish-brown pigment bands appeared consistently on the chromato-

Table 3—The relative quantity of individual anthocyanins in strawberry varieties.[1]

Variety	Pigment No. 1	2	3	4	5	6
Acadia	+	++++	<+	++	+	+
Gorella	+	++++	<+	++	+	<+
K60-98	+	++++	<+	+++	+	±
K63-280	+	++++	±±±	++	±	±
Midway	+	++++	<+	++	+	<+
NJ 857	+	++++	+	++	±	<+
Redcoat	+	++++	+	+++	+	+
Senga Sengana	+	++++	++	+++	±	±
Sparkle	+	++++	+	++	<+	<−

[1] The relative quantity of the pigments was estimated visually and indicated as follows: ++++ = major, +++ = intermediate, ++ = minor, + = trace quantities. The best sources for the unidentified pigments are underlined.

Table 4—The relative quantity of individual anthocyanins in rhubarb varieties.[1]

Variety	Pigment No. 1	2	3
		%	
German Wine	33	66	1
Sunrise	48	51	1
Sutton's Seedless	48	50	2
Valentine	58	41	1

[1] The relative quantity of the pigments was measured spectrophotometrically. The OD measurements were carried out at 527, 533 and 525 mm respectively. The solvent was 1% HCl in MeOH.

grams. One of these pigments moved approximately with the same speed as the No. 3 anthocyanin when the chromatogram was developed with either BBFW or BFW. The slower moving reddish-brown band had an av R_{Cy} value of 32 and 45 in BBFW and BFW respectively. The color of these bands and their reactions with chromogenic reagents indicated that they were not anthocyanins.

Red Radish

The radish anthocyanins were resolved into thirteen bands by the solvents used in this survey. Eight of these pigments appear to be previously unreported (Table 2). The presence of two acylated pigments (pelargonidin 5-glucoside, 3-sophoroside acylated with p-coumaric or ferrulic acid) is well established (Harborne et al., 1957). According to Ishikura et al. (1963), three additional acylated pelargonidin 5-glucoside, 3-diglucoside are found in red radish. This is supported by Fouassin (1956) who also detected five acylated pelargonidin derivatives. The anthocyanin composition of purple radish varieties is similar to that of the red varieties but cyanidin replaces pelargonidin as the aglycon moiety of the pigments (Harborne, 1963).

The color of the anthocyanin bands indicated that all of them are pelargonidin glycosides. Two very faint anthocyanin bands were observed, in addition to those listed in Table 2, on some chromatograms. One of them appeared between the No. 6 and 7 bands, and the other between the No. 12 and 13 bands. The tentative identification of the anthocyanin bands was difficult because of the large number of pigments present. It was assumed that the most concentrated pigment bands were identical with Raphanusin A and B (pelargonidin 5-glucoside, 3-sophoroside acylated with p-coumaric and ferrulic acid respectively), the presence of which was most frequently reported in the literature (Table 1).

The R_f values given by Ishikura et al. (1963) for the radish anthocyanins were also taken into consideration when the pigments identified by these authors were assigned to the bands appearing on the chromatograms. The qualitative and quantitative anthocyanin patterns of the examined varieties were not noticeably different.

Onion

Five previously unnoticed anthocyanins were found in addition to the three pigments reported in the literature. Little information was available from the literature on the anthocyanin composition of the purple onion varieties. Robinson et al. (1932) found cyanidin 3-pentoseglycoside while Fouassin (1956) detected three cyanidin derivatives. Brandwein (1965) identified peonidin 3-arabinoside in the Southport Red Globe variety. This is the only report of a peonidin glycoside occurring in the Liliaceae family (Harborne, 1967a). Since there was a definite discrepancy between the R_f values reported by this author for the onion anthocyanin and that of the literature values for peonidin 3-arabinoside, we had reservations accepting this identification.

Chromatographic analysis revealed the presence of eight anthocyanins in the onion varieties examined. It was assumed that the presence of the most abundant anthocyanins was noted by Fouassin (1956). The R_f values reported by this author were also taken into consideration, when these "known" pigments were assigned to anthocyanin bands appearing on the chromatograms (Table 2). All pigments appeared to be cyanidin derivatives with the exception of the No. 3 anthocyanin. The color of the pigment and its reactions with chromogenic reagents indicated that this pigment is a peonidin derivative. Cochromatography of the No. 3 onion anthocyanin with authentic peonidin 3-arabinoside prepared from cranberry (Zapsalis et al., 1965) revealed that the two pigments are definitely not identical.

In addition to the pigments listed in Table 2, there was some indication of two more anthocyanins occurring in very small quantities. A pigment with R_{Cy} value of 16 and 11 in BBFW and BFW, respectively, was detected on some chromatograms. Two papers developed with BBFW showed the presence of a fast moving anthocyanin (R_{Cy} = 297). It should be noted that this pigment moved considerably faster than authentic peonidin 3-arabinoside (R_{Cy} = 216).

The difference between the pigment composition of the outer dry scales and the fleshy scale-leaves appears to be quantitative rather than qualitative. The Nos. 2 and 5 pigments were more abundant in the fleshy scale-leaves than in the dry scales. This might indicate that these pigments are more labile than the others present, and as a result of this their relative quantities decrease as the sap is withdrawn from the outer scale-leaves during maturation. The qualitative and quantitative anthocyanin patterns of the examined onion varieties were not noticeably different.

In estimating the number of anthocyanins in the particular plant extract the possibility of anthocyanin bands occurring as artifacts was considered. Acid hydrolysis and deacylation of anthocyanins can occur during extraction and concentration of the pigments (Harborne, 1958). Acid hydrolysis can lead to the formation of anthocyanidins. Since the aglycones move faster in a butanolic solvent system than the anthocyanins, they were easily distinguished from the anthocyanins. Anthocyanidin bands were observed on the chromatograms occasionally, but these bands were not listed in Table 2. Anthocyanins containing di- or tri-saccharide and/or acyl group in their structure can also give intermediates on acid hydrolysis.

Under the carefully controlled conditions of the experiment only a small fraction of the pigments could be hydrolyzed. This precluded that the intermediates of minor anthocyanins appeared on the chromatograms as "new" bands because their quantities would have been below the threshold of detection. An examination of Table 2 shows that only the major radish pigments could give intermediates appearing as "new" anthocyanin bands. To discount this possibility, the results were verified by pressing the anthocyanin containing sap from the scraped, frozen and thawed epidermal layer of red radishes. The sap was applied directly on Whatman No. 3 paper and developed with BFW. The chromatographic pattern obtained was similar to that reported in Table 2 for radish. This showed that the "new" pigment bands in radish did not occur as intermediates on acid hydrolysis of the anthocyanins.

According to Harborne (1967b) some phenolics form loose complexes with each other and may give rise to multiple spots. However a thorough search of the literature failed to reveal experimental evidence which would indicate that this phenomenon occurs with anthocyanins. This was not unexpected considering that the naturally occurring copigment-metal-anthocyanin complexes readily decompose at low pH and they could be isolated only in the absence of added acid (Bayer, 1958; Asen et al., 1967; Yazaki et al., 1967). Multiple spot formation due to complex formation apparently could not have occurred under the highly acidic conditions which prevailed during extraction and chromatography.

Cinnamic acids and their derivatives exist in solution as an equilibrium mixture of *cis* and *trans* isomers (Williams, 1955), which can result in multiple spot formation. Since radish contains a number of acylated anthocyanins this possibility was also considered. Isomerization of the acylated pigments could have occurred during extraction and concentration. These isomers are not separated by butanolic solvent systems (Harborne, 1967b), therefore the isomerization of acylated anthocyanins could not account for the appearance of "new" pigment bands.

Since the presence of a great number of previously unreported anthocyanins was revealed in this survey, it is appropriate to discuss the factors which contributed to this. Most of the "new" pigments were present in relatively small quantities. This indicates that a highly sensitive technique

was employed in our survey, which decreased the level where the anthocyanins could be detected. The sensitivity of the chromatographic method was increased by the following factors:

1. A heavy grade paper (Whatman No. 3 MM) was used instead of the customary Whatman No. 1 paper. This allowed for the application of a sufficiently large amount of pigment extract where the concentration of trace pigments reached the level of detectability.
2. The developing solvents BBFW and BFW improved the resolution and reduced the diffusion of the pigment bands. The new solvent system (BFW) is slightly slower, but it gives more compact bands and better resolution than the commonly used 1-butanol-acetic acid-water (4:1:5).
3. Only the anthocyanin rich tissues of the examined plant material were used for the preparation of the pigment extracts. This gave a fairly concentrated extract which reduced the need for an excessive further concentration.

It should be realized that the number of anthocyanins present in the plant material included in this survey is possibly greater than reported in this article. As indicated in the text, some of the chromatograms suggested that some of the bands may have consisted of more than one pigment. It is also possible that pigments escaped detection because their concentration was below the threshold of detection.

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Enzyme-Catalyzed Reactions as Influenced by Inert Gases at High Pressures

SUMMARY—The activities of tyrosinase, invertase, trypsin and chymotrypsin were studied after exposure to one or more of the following high-pressure inert gases: N_2O at 600 psig or N_2 or Ar each at 5000 psig. Exposure to high-pressure nitrogen or argon failed to significantly inhibit the rate of tyrosinase activity in fluid systems. However, the rates of tyrosinase-catalyzed reactions in shell-cast gelatin gels were significantly depressed by exposure to high-pressure nitrogen, and even more so by high-pressure nitrous oxide. This inhibition proved to be oxygen dependent and reversible. Pressurization experiments with invertase, trypsin and chymotrypsin indicated that high-pressure N_2O did not significantly inhibit these enzymes. This lends support to the hypothesis that high-pressure inert gases inhibited tyrosinase activity in nonfluid systems by decreasing the availability of oxygen, rather than by physically altering the enzyme. It must be concluded that there is little hope that the enzymes in food systems can be effectively inhibited by brief exposure to inert gases at pressures of 5000 psig or less.

INTRODUCTION

SCHREINER (1965) reported that tyrosinase in a simple aqueous system could be inhibited by inert gases as superatmospheric pressures. This finding stimulated initiation of the present study to determine if inert gases temporarily applied at moderately high pressures could be used to control enzyme activity in food systems.

It is important to distinguish between the effects of hydrostatic and nonhydrostatic (gaseous) pressure. The effects of hydrostatic pressure on microorganisms (Heden, 1964; Hite et al., 1914) and enzymes (Curl et al., 1950; Suzuki et al., 1963) have been explored extensively. Reactions with a positive ΔV (activation volume, defined as volume of products minus volume of reactants) are decelerated by high pressure, whereas reactions with a negative ΔV (products occupy less volume than reactants) are accelerated by high pressure (Suzuki et al., 1963). The effects of hydrostatic pressure are often not evident until pressures of 1000 to 5000 atm have been applied (Johnson et al., 1954). Pressures of this magnitude could, however, have an undesirable effect on the texture of food tissue.

High-pressure inert gases such as N_2, N_2O, He, Ne, Ar, Kr and Xe have been studied to determine their effects on biological systems. Studies of this type have included the growth rate of the mold *Neurospora crassa* (Schreiner et al., 1962; Schreiner et al., 1963; and Buchheit et al., 1966), the growth of protozoans (Sears et al., 1961), the oxygen dependent radio sensitivity of plants (Ebert et al., 1958), the growth rate of mammalian epithelial cells (Bruemmer et al., 1967) and the activity of tyrosinase (Schreiner, 1965).

To explain the effects of inert gases on oxygen dependent systems, Ebert et al. (1958) proposed that chemically inert gases compete with oxygen molecules for cellular sites, and that the extent of oxygen displacement (at constant total pressure) depends on the ratio of oxygen to inert gas. If this proposed mechanism is correct, and this is the only mode of action of inert gases, then there would be no obvious reason to suspect that inert gases would inhibit nonoxygen dependent reactions. Preliminary studies by Doebbler (1967 personal communication) suggest that inhibition does occur for some nonoxygen dependent enzymic reactions.

If enzymes in general could be irreversibly inactivated or inhibited by short-time application of inert gases at moderately high pressures, this would have considerable value in the food industry. This method could be used to control enzyme activity in food preserved by ionizing radiation, or to inhibit enzyme activity in products sterilized by high temperature-short time (HTST) techniques.

The present study was done to explore the possibility of controlling some food-related enzymes by the brief application of inert gases at high pressure. One oxidative and several hydrolytic enzymes were selected for investigation. Tyrosinase and alpha-chymotrypsin were specifically chosen because of their use in previous investigations by Schreiner (1965) and Doebbler (1967 personal communication). The inert gases N_2O, Ar and N_2 were selected for the same reason. Studies with N_2O were limited to 600 psig since slightly higher pressures resulted in liquefaction and damage to the gas compressor. Argon and nitrogen were studied at 5000 psig (ca. 341 atm) so as to exceed by a considerable margin the pressures at which Schreiner (1965) observed inhibitory effects.

EXPERIMENTAL

Equipment

The high-pressure apparatus shown in Figure 1 consisted of (1) an air-operated piston compressor (Model AC-15, Pressure Products Industries, Hatsboro, Pa.), (2) a high-pressure reaction vessel (Model PJE-3-300, Pressure Products Industries, Hatsboro, Pa.) 300 ml capacity, austenitic cold-forged type 316 stainless steel, 30,000 psig rating, with agitation provided by pendulum type motion, and (3) accessory gauges, valves, tubing and a constant temperature bath.

Effect of pressurization time on tyrosinase activity

Tyrosinase activity was determined by measuring dopachrome absorption at 470 mμ (Boscan et al., 1962). A mixture of 28.0 ml L-tyrosine (0.001*M*, Nutritional Biochemicals Co.), 20.0 ml phosphate buffer (pH 7.2, ionic strength 0.05) and 8.0 ml distilled water was placed in the open chamber of the high-pressure reaction vessel and equilibrated at 20°C. An 8.0 ml volume of tyrosinase solution (500 units tyrosinase/3.0 ml phosphate buffer, Sigma Chemicals Co.; grade II, 500 units/mg, where a unit is defined as equal to Δ Absorbance 0.001/minute, 3.0 ml, 280 mμ, pH 6.5, 25°C) was added to the mixture and this moment was regarded as zero time.

The ratio of enzyme to substrate was previously determined to yield a zero order reaction with respect to substrate concentration. The vessel was closed and the sample was maintained at 20°C while argon or nitrogen gas was introduced (superimposed over the normal atmosphere) for approximately 3 min to increase the pressure to 5000 psig. The vessel was agitated for five sec following attainment of the desired pressure.

Aliquots of 6.4 ml were periodically collected in a tube containing 2.0 ml acetate buffer (pH 4.6, 0.2 M) by opening a valve (Part J, Fig. 1) connected to a dip tube. Absorbance at 470 mμ was measured using a Spectronic 20 colorimeter (Bausch and Lomb Optical Co.). After samples which had been pressurized for various times were analyzed, a plot was made of absorbance vs. time. The slopes and 95% confidence limits were calculated using linear regression analysis (Draper et al., 1966).

Control samples were prepared and handled in the same manner as high-pressure

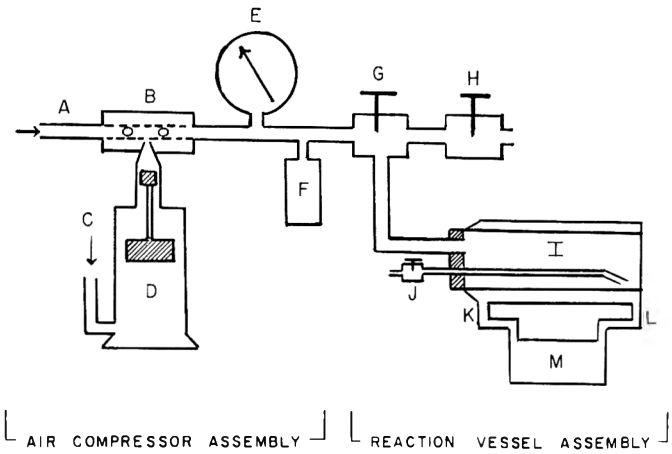


Fig. 1—High-pressure apparatus. A. Inlet, gas; B. valve, one way, check; C. air, for compressor operation; D. compressor, air operated; E. gauge, pressure; F. reservoir, gas, 90 ml capacity; G. valve, 3-way; H. valve, 2-way; I. reaction vessel, 300 ml capacity; J. valve, sampling, with dip tube; K. inlet, water jacket; L. outlet, water jacket; M. bath, constant temperature liquid with circulating pump.

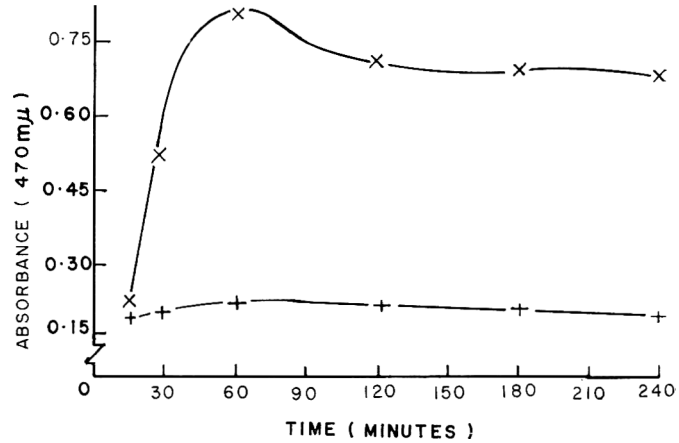


Fig. 2—The effect of acetate buffer on trosinase activity and dopachrome stability. After 15 min reaction time, 20.0 ml of acetate buffer (pH 4.6, 0.2 M) were added. The initial reaction mixture contained 28.0 ml L-tyrosine (0.001 M), 20.0 ml phosphate buffer (pH 7.2, ionic strength 0.05), 8.0 ml distilled water and 8.0 ml tyrosinase solution (500 units tyrosinase per 3.0 ml phosphate buffer).

samples, except they were reacted in an atmosphere of normal composition and pressure and a pipette rather than a dip tube was used to collect aliquots from the open reaction vessel.

Since several minutes elapsed between sample collection and measurement of absorbance, it was desirable that dopachrome absorbance remain constant during this interval. Concern over this point arose because of: (1) possible damage to the enzyme (surface denaturation) during dip tube sampling, and (2) the loss of oxygen that would occur when pressure was released from the sample. Both events would cause the pressurized samples to exhibit less activity than the controls during the interval between sampling and measurement of absorbance. Figure 2 shows that acetate buffer was effective in maintaining a quite constant absorbance at 470 mμ for the period in question, thereby minimizing the chances that the above factors could affect the results. Further experimentation also established that dopachrome red was stabilized by the acetate buffer treatment, i.e., the system was static in the sense that maintenance of dopachrome at a constant level was not achieved by a mechanism involving equal rates of formation and oxidation.

Influence of exposure to N_2 for 15 min at 5000 psig

Gelatin gels were chosen as a nonfluid system since gelatin did not interfere with the colorimetric analysis for dopachrome. Shell-cast gels, rather than bulk gels were used because preliminary observations showed that penetration of high-pressure gases into bulk gels required excessive time for equilibration.

A 1.58 g quantity of gelatin (Swift's Velvatek, 250 Bloom, Gelatin Dep't., Swift and Co., Chicago) was slowly added with constant magnetic stirring to 16.0 ml of boiling distilled water. A mixture of 7.0 ml L-tyrosine solution (0.002*M*) and 5.0 ml phosphate buffer (pH 6.85, ionic strength 0.10) was added and the entire system was allowed to cool to 30°C. A 2-ml volume of tyrosinase solution (500 units tyrosinase/3.0 ml phosphate buffer) was added to the mixture and this moment was regarded as zero time. This ratio of enzyme to substrate yielded a zero order reaction with respect to substrate concentration.

The entire suspension was then poured into a large glass test tube (38 × 200 mm) and the tube was positioned horizontally in an ice-water bath and rotated about its major axis for three min. at 60 rpm. A clear, thin shell-gel approximately 20 mm deep was produced on the inner surface of the tube. The tube was then removed from the rotator and placed in the high-pressure reaction vessel which was maintained at 15°C. Nitrogen (5000 psig) or nitrous oxide (600 psig) was then introduced while the sample temperature was carefully maintained at 15°C both during and following the pressurization period.

After a 15-min treatment, the pressure was released, the test tube was removed, and the shell-gel was melted by heating to 35°C for 5 min. This melting procedure had no detectable effect on tyrosinase activity. The melted gel was poured into a cuvette and maintained at 25°C while absorbance at 470 mμ was periodically measured. Samples designated as "agitated" were stirred for 30 sec to incorporate air prior to each absorbance reading.

The control samples were prepared and treated in the same manner. Pressurization was omitted.

Influence of exposure to N_2O at 600 psig in fluid systems

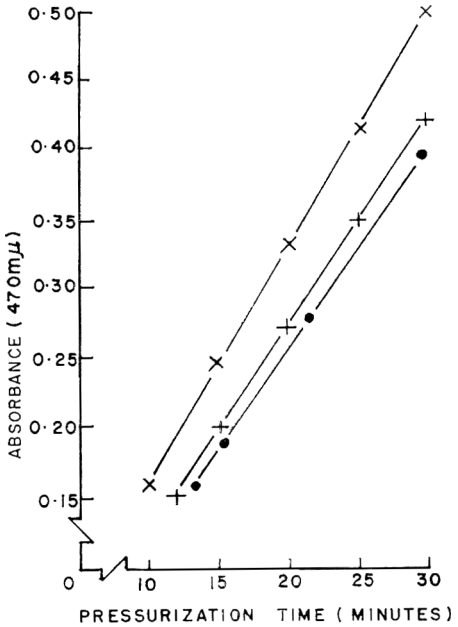
A mixture of 40.0 ml acetate buffer (0.2*M*, pH 4.6) and 8.0 ml sucrose solution (15.33%, w/v) was added to 1.0 ml invertase solution. The invertase solution consisted of 0.4 mg dry yeast invertase (grade B, Cal Biochem, Los Angeles) 0.9 ml water, 0.065 ml glycerol and 0.035 ml citrate buffer at pH 4.45.

The combination was poured into the chamber of the high-pressure vessel (Part I, Fig. 1), which was maintained at 15°C. Nitrous oxide (600 psig) was introduced, and the sample temperature was carefully maintained at 15°C both during and following the pressurization period. After 60 min the pressure was slowly released to avoid any detectable decrease in sample temperature, the vessel was opened (designated as zero time) and 1.0 ml aliquots were periodically pipetted from the solution and analyzed for sucrose hydrolysis by the method of Schaffer et al. (1933).

The control samples were prepared and treated in the same manner as the high-pressure samples, except they were reacted in an atmosphere of normal composition and pressure.

Influence of exposure to N_2O at 600 psig in nonfluid systems

A 1.0 g quantity of agar (Difco Bacto Agar, B140, Detroit) was slowly added with constant stirring to a mixture of 40.0 ml acetate buffer (0.2*M*., pH 4.62) and 8.0 ml sucrose solution (15.33%, w/v). The mixture was heated to boiling, cooled to 37°C, and 1.0 ml of invertase solution (as before) was added. Invertase suffered no detectable loss in activity when briefly subjected to temperatures of 37°C. The entire mixture was poured into a large glass tube and a shell-gel was formed. The tube was then placed in the high-pressure reaction vessel, which was maintained at 15°C, and the sample was treated with 600 psig N_2O as described for the fluid invertase samples. After 60 min the pressure was slowly released and the vessel was opened (designated as zero time). Portions of the gel were periodically removed



× = atmospheric controls:
y = 0.0169 × −0.0099
slope = 0.0169 ± 0.0006 (95% C.L.)
$\bar{y}$ = 0.330 ± 0.006 (95% C.L.)
\+ = samples exposed to nitrogen at 5000 psig:
y = 0.0150 × −0.0224
slope = 0.0150 ± 0.0029 (95% C.L.)
$\bar{y}$ = 0.275 ± 0.025 (95% C.L.)
● = samples exposed to argon at 5000 psig:
y = 0.0148 × −0.0421
slope = 0.0148 ± 0.0034 (95% C.L.)
$\bar{y}$ = 0.280 ± 0.015 (95% C.L.)

Fig. 3—Influence of pressurization time (N_2 or Ar at 5000 psig) on tyrosinase activity in fluid systems analyzed immediately following depressurization.

and 1.00 g quantities were analyzed for sucrose hydrolysis by the method of Schaffer et al. (1933).

The control samples were prepared and treated in the same manner as the high-pressure samples, except they were reacted in an atmosphere of normal composition and pressure.

Influence of pressurization time on trypsin activity

A 7.5 ml volume of trypsin (Nutritional Biochemicals Corp., Cleveland, salt-free and 2× crystallized from ethanol, 250 μg/ml $10^{-3}N$ HCl) was added to a mixture of 42.5 ml phosphate buffer (pH 7.2, ionic strength 0.10) and 50.0 ml casein solution (Nutritional Biochemicals Corp., Cleveland 0.5 gm casein/50 ml phosphate buffer) at 25°C. This moment was designated as zero time. A 70.0 ml portion was poured into a large glass tube, which was then placed inside the high-pressure chamber maintained at 25°C. Pressurization with 600 psig N_2O and sampling by means of a dip tube were conducted as previously described. Discharged samples were agitated to remove N_2O bubbles and the reaction was stopped by the addition of an equal volume of 5% trichloroacetic acid (TCA). The entire sample was then vacuum filtered through a sintered glass filter (medium porosity) and the absorbance of the clear filtrate (due to tyrosine and tryptophane) was determined at 280 mμ with a recording spectrophotometer (model DK-2, Beckman Instruments, Inc.) in general accordance with the method of Rick (1965b).

Control samples were prepared and treated exactly the same, except they were allowed to react in an atmosphere of normal composition and pressure.

Influence of pressurization time on alpha-chymotrypsin activity

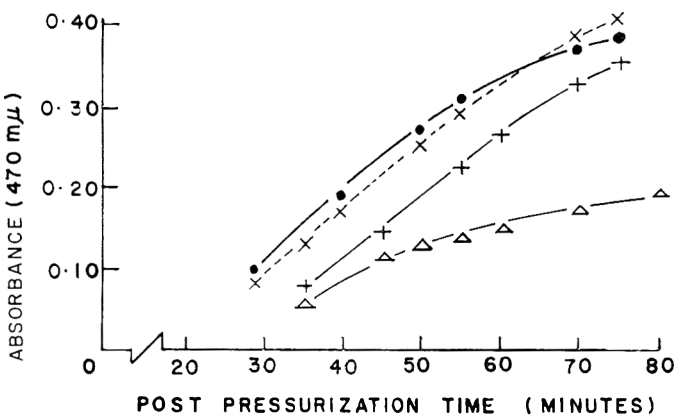
A 5.0 ml volume of alpha-chymotrypsin (Nutritional Biochemical Corp., Cleveland, salt-free and 3X crystallized from ethanol, 100 μg/ml $10^{-3}N$ HCl) was added to a mixture of 45.0 ml phosphate buffer (pH 7.2, ionic strength 0.10) and 50.0 ml casein solution (as before) at 25°C. This moment was regarded as zero time. From this point on, the degree of proteolysis for both the pressure and the control samples was measured exactly as described for trypsin analysis and outlined by Rick (1965a). The slopes (Δ A 280 mμ/minute) and their 95% confidence limits were calculated using linear regression analysis (Draper et al., 1966).

RESULTS & DISCUSSION

Influence of pressurization time on tyrosinase activity

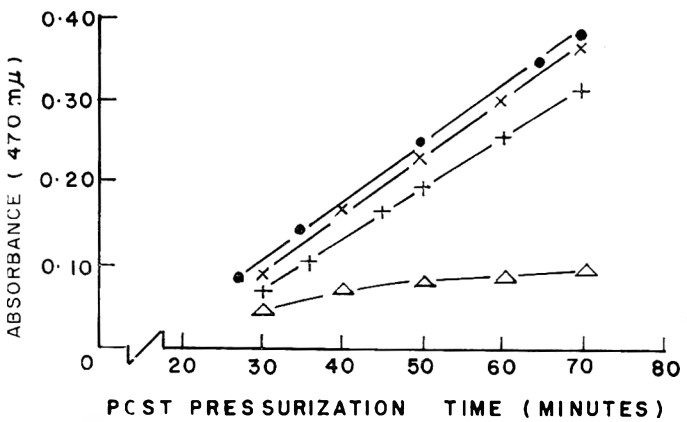
The effects of high-pressure argon or nitrogen (various exposure times) on tyrosinase activity in fluid aqueous systems are shown in Figure 3. Each point represents duplicate samples pressurized for the time indicated, and then analyzed at atmospheric pressure. Neither nitrogen nor argon at 5000 psig (340 atm) significantly reduced the rate of reaction (ΔA 470 mμ/minute) as compared to the atmospheric control. Both gases caused a significant downward displacement of the absorbance-time curve as compared to the control curve. Initial imposition of a high gas pressure (during the first 10 min) had an inhibitory effect on tyrosinase, but continued pressurization beyond ten min. had no additional inhibitory effect. Absorbance of the pressurized sample was always less than the control.

The results of Figure 3 are in partial disagreement with those of Schreiner (1965). At a partial pressure of 30.6 atm (compared to 340 atm in the present study) Schreiner reported that the reaction rate of tyrosinase was reduced 32%



Symbol	Meaning	Slope (△ A 470 mμ/min)
×	agitated, atmospheric control	0.0072
●	nonagitated atmospheric control	0.0072
+	agitated, 5000 psig N_2 sample	0.0070
△	nonagitated, 5000 psig N_2 sample	0.0023

Fig. 4—Influence of exposure to N_2 for 15 minutes at 5000 psig on tyrosinase activity in nonfluid systems analyzed at various times following depressurization.



Symbol	Meaning	Slope (△ A 470 mμ/min)
×	agitated, atmospheric control	0.0065
●	nonagitated, atmospheric control	0.0065
+	agitated, 600 psig N_2O	0.0060
△	nonagitated, 600 psig N_2O	0.0010

Fig. 5—Influence of exposure to N_2O for 15 minutes at 600 psig on tyrosinase activity in nonfluid systems analyzed at various times following depressurization.

by argon and 40% by nitrogen as compared to a control at atmospheric pressure.

Influence of exposure to N_2 for 15 min at 5000 psig on tyrosinase activity

The effect of 15-min. exposure to nitrogen at 5000 psig or nitrous oxide at 600 psig on tyrosinase activity in gelatin shell gels is shown in Figures 4 and 5.

Each figure represents results of a typical experiment conducted by pressurizing a single sample, releasing the pressure, melting the gel and measuring absorbance at various post-pressurization times. Several conclusions can be drawn from these data.

Effect of pressurization. In all instances, treatment with either high-pressure N_2 or N_2O resulted in inhibition of tyrosinase activity. For the unagitated pressurized samples, this is evident from their smaller initial absorbance values (35 min. values for N_2 data and 30 min values for N_2O data) and their lesser slopes as compared to the unagitated controls. For the agitated pressurized samples this inhibition is evident from the downward displacement of their absorbance curves as compared to the agitated controls.

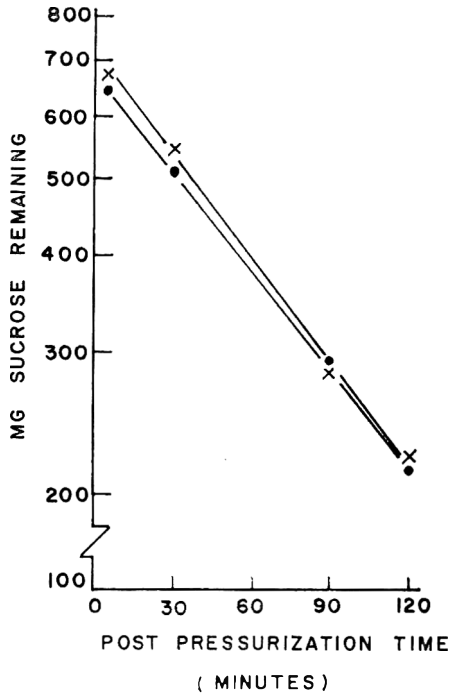
Agitation following pressure release. Agitation of samples following release of the pressure greatly enhanced the activity of tyrosinase in the pressure-treated samples, but agitation of the control samples had no effect. This is to be expected since the pressurized samples would be depleted of oxygen by the pressurization-depressurization process (release of the pressure would remove considerable oxygen along with inert gases), whereas the control samples would be in a state of near equilibrium with air throughout the reaction period.

The fact that agitation (aeration) restored the depressurized samples to the same activity as the controls, indicates that tyrosinase inhibition was likely caused by the ability of high-pressure gases to limit the availability of oxygen rather than by any pressure induced changes in tyrosinase. Tissue systems would no doubt behave like the unagitated samples.

N_2 vs. N_2O. Nitrous oxide at 600 psig inhibited tyrosinase to a greater extent than nitrogen at 5000 psig. The reaction rate (slope) of the nonagitated sample treated with 600 psig N_2O was only one sixth that of the nonagitated control sample (Fig. 5), whereas the reaction rate of the nonagitated sample treated with 5000 psig N_2 was one third that of the nonagitated control sample (Fig. 4).

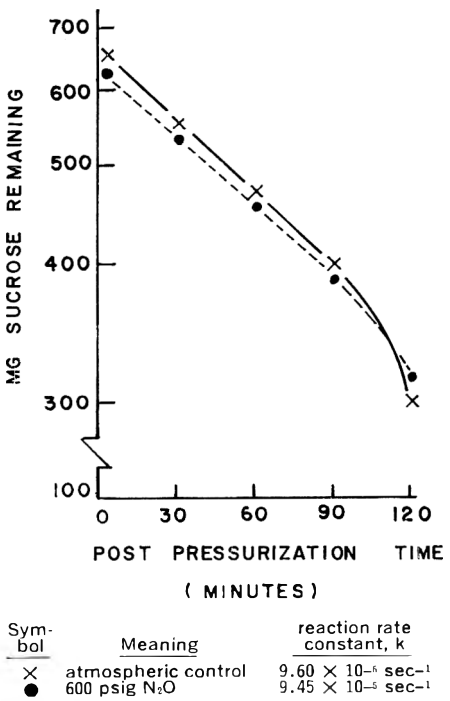
Influence of exposure to N_2O on invertase activity in fluid systems

The effect of N_2O at high pressure on invertase activity in a fluid aqueous system was determined in duplicate by exposing the system to 600 psig N_2O for 60 minutes, releasing the pressure and measuring absorbance at various post-pressurization times. From the results shown in Fig. 6 it is obvious that the N_2O treatment had no significant effect on invertase activity.



Symbol	Meaning	reaction rate constant, k
×	atmospheric control	16.0×10^{-5} sec^{-1}
●	600 psig N_2O	15.5×10^{-5} sec^{-1}

Fig. 6—Influence of exposure to N_2O for 60 minutes at 600 psig on invertase activity in fluid systems analyzed at various times following depressurization.



Symbol	Meaning	reaction rate constant, k
×	atmospheric control	9.60×10^{-5} sec^{-1}
●	600 psig N_2O	9.45×10^{-5} sec^{-1}

Fig. 7—Influence of exposure to N_2O for 60 minutes at 600 psig on invertase activity in nonfluid systems analyzed at various times following depressurization.

Influence of exposure to N_2O on invertase activity in nonfluid systems

The effect of N_2O on invertase activity in agar shell-cast gels was determined in duplicate by exposing the samples to 600 psig N_2O for 60 min, releasing the pressure, and analyzing liquefied aliquots of the gel at various times following depressurization. The results in Figure 7 clearly demonstrated that 600 psig N_2O had no inhibitory effect on invertase activity. Invertase, unlike tyrosinase, does not utilize molecular oxygen as a substrate. Any theories postulating competition between inert gas molecules and substrate oxygen molecules for reactive sites on the enzyme surface are not applicable to invertase.

Influence of pressurization time on trypsin activity

The effect of N_2O on trypsin-catalyzed hydrolysis of casein in fluid aqueous systems was determined in duplicate by exposing the system to 600 psig N_2O and then drawing samples for analysis at various times. From the results shown in Figure 8, the pressure treatment had no detectable effect on the activity of trypsin in aqueous solution. Since no inhibitory effect was achieved using N_2O (most effective of the inert gases in the case of tyrosinase), investigation of the other inert gases was not considered worthwhile.

Influence of pressurization time on alpha-chymotrypsin activity

The effect of N_2O on alpha-chymotrypsin-catalyzed hydrolysis of casein in a fluid aqueous system was determined in triplicate by exposing the system to 600 psig N_2O and then drawing samples for analysis at desired intervals. The results in Figure 9 show a tendency for the pressure treatment to depress the reaction rate (ΔA 280 mμ/minute) as compared to the atmospheric control, however, the difference was not statistically significant at the 95% confidence level.

Additional studies were conducted in duplicate for pressurization times ranging from 60 to 180 min, since it appeared from Figure 9 that more prolonged pressurization might result in a significant rate depression. The 60–180-min data for the control and pressurized samples resulted in parallel lines, with the line for the pressurized samples being displaced downward from the control by a distance that was not statistically significant. N_2O at 600 psig had no significant inhibitory effect on the activity of alpha-chymotrypsin in aqueous solution.

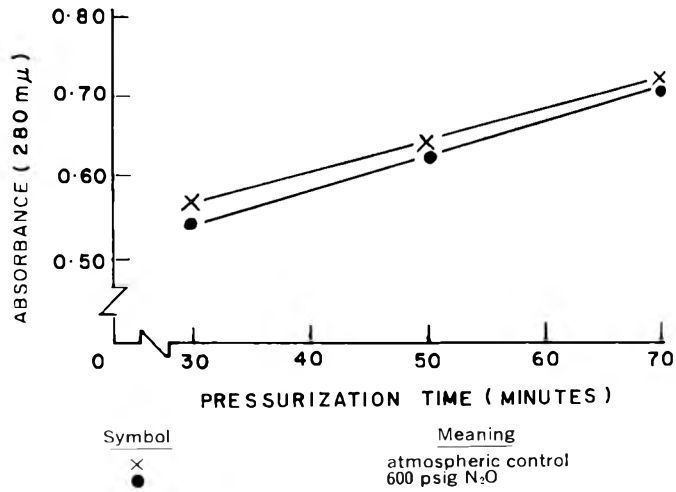
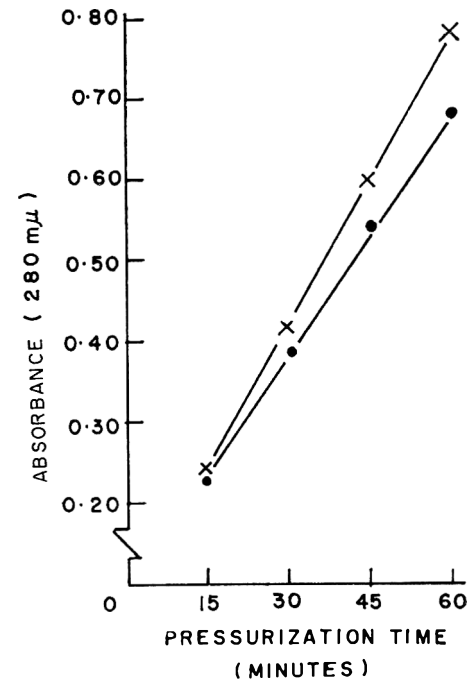


Fig. 8—Influence of pressurization time (N_2O at 600 psig) on trypsin activity in fluid systems analyzed immediately following depressurization.



Symbol	Meaning	Slope (△ A 280 mμ/min) and 95% confidence limit
×	atmospheric control	1.21 ± 0.31
●	600 psig N_2O	1.02 ± 0.27

Fig. 9—Influence of pressurization time (N_2O at 600 psig) on alpha chymotrypsin activity in fluid systems analyzed immediately following depressurization.

CONCLUSIONS

HIGH-PRESSURE treatments with gases such as N_2, Ar and N_2O significantly inhibited the activity of the oxygen dependent enzyme tyrosinase, but did not significantly alter the activities of invertase, trypsin or chymotrypsin.

Two apparently different types of inhibition were observed with tyrosinase. One type of inhibition was evident in the fluid tyrosinase samples where the absorbance curve for the pressurized samples was displaced downward from that of the controls. A possible explanation for this occurrence is that the imposition of the pressure resulted in temporary enzyme inhibition followed by rapid restoration of normal activity as the pressure was held constant at the desired level. This suggestion, although unattractive from a theoretical standpoint, is compatible with the results of Figure 3.

Alternatively, it might be suspected that some consistent error in handling the pressurized samples resulted in their fixed lower absorbance as compared to control samples at corresponding reaction times. An obvious possibility is that the samples continued to react during the few minutes between pressure release and analysis, with the pressure-treated samples reacting more slowly than the controls. For example, the dip tube sampling technique could have resulted in temporary temperature changes, partial denaturation of tyrosinase and oxygen loss from the sample. Considerable care was taken immediately after sampling to assure that the reaction was stopped, pertinent products were stabilized and the sample temperature was controlled.

It was also proved experimentally that the dip tube sampling method caused no detectable change in tyrosinase activity. In spite of these considerations, it must be remembered that only presumptive evidence exists for inhibition of tyrosinase in liquid systems during imposition of pressure. An undetected constant error of some sort caused the pressure-treated fluid tyrosinase samples to exhibit lower absorbance values than the controls.

The second type of tyrosinase inhibition was observed in the unagitated gel samples of Figures 4 and 5. The rates of reaction (slope of the lines) were significantly less in the pressure-treated samples (following release of the pressure) than in the controls. This inhibition was oxygen dependent and reversible, as is evident from the fact that the pressure-treated samples could be restored to a normal activity by aeration. Oxygen was depleted in the sample as the pressure was released. The only significant effect of high-pressure gases as used in this study was to interfere with oxygen availability.

This study involved only a few enzymes, each at a single set of conditions. Before abandoning the hope that high pressures can be used to control enzyme activity, it would be advisable to determine the extent to which the effectiveness of a pressure treatment is influenced by such variables as temperature, pH and concentration of the various constituents. It would be desirable to test other enzymes, and pressures greater than 5000 psig. Hydrostatic pressures on the order of 5000 atm (ca. 75,000 psig) can result in partial inactivation (irreversible) of some enzymes, it would seem especially worth-while to investigate the pressure range 5000 to 75,000 psig using inert gases (Curl et al., 1950; Miyagawa et al., 1963; Suzuki et al., 1963).

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The Influence of Tension on Pre-Rigor Excised Bovine Muscle

SUMMARY—The influence of four levels of tension in two pre-rigor excised bovine muscles on fiber diameter, sacromere length, percent kinkiness, and shear value were studied. Fiber diameter was found to be smaller with 1,000-g pull treatment than when there was no tension, but no significant change was found with succeeding increases in tension. Sarcomere length was found to increase to the 2,500 g pull treatment and then level off. Both muscles exhibited less kinkiness to the 1,000-g pull treatment. However, after this level the semitendinosus muscle increased whereas the semimembranosus decreased. In like manner, a considerable decrease was noted in shear force to the 1,000-g pull treatment for both muscles, but after this point a small increase was noted for the semitendinosus muscle whereas the semimembranosus muscle did not change.

INTRODUCTION

COMMONLY ASSOCIATED differences in meat tenderness among muscles—age, breed, sex, aging, etc.—have been largely attributed to the quality and quantity of the connective tissues. However, muscle fibers, which account for approximately 75% of the muscle mass, are also very important.

The work of Hiner et al. (1953), Tuma et al. (1962), Herring et al. (1965b) and Reddy et al. (1967) indicated there was a negative relationship between fiber diameter and tenderness. As fiber diameter increased, to a point, there was a corresponding decrease in tenderness. Experiments conducted by Locker (1960), Herring (1965a) and Marsh et al. (1966) suggested that the state of contraction of the muscle may be related to tenderness and that muscles that are in a fully or partially contracted state are less tender than those that have not contracted.

Both muscle fiber diameter and state of contraction vary inherently and must be considered in any evaluation of tenderness. Fiber size has been found to vary between the major animal classes (Mayeda, 1890) as well as between muscles of the same animal (Hammond et al. 1932). In a study by Joubert (1956), differences in fiber diameter were shown to exist between breeds and sexes. Maturity, as reported by Henrickson et al. (1963), caused an increase in fiber size. Fiber size increases rapidly while the animal is quite young and tends to level off as the animal approaches maturity.

Yeates (1964) reported that the level of nutrition affected fiber size. The experiment revealed that, with starvation of the adult animal, shrinkage in cross-sectional areas of the muscles was associated with reduction in fiber diameter. However, with recovery of live weight, both whole muscle dimension and muscle fiber diameter appeared to return to normal.

The state of muscular contraction is primarily a result of the postmortem changes that are both natural and induced. The most pronounced physical change in muscle extensibility results from the occurrence of rigor mortis. As rigor approaches, the muscle goes from a highly extensive elastic condition to the inextensible and rigid condition of the muscle in full rigor. Along with this change in extensibility, Locker (1960) indicated there was a gradual shortening of the sarcomere as rigor approached, which leaves the muscle in a semicontracted state.

The effect of temperature on the contractile state of the muscle has been shown to have considerable influence. Locker et al. (1963) showed that shortening of the muscle fiber occurred when exposed to very cold temperatures. Herring et al. (1965a) reported that slightly more shortening appeared, as indicated by sarcomere length, to take place in stretch-restrained muscle at 1°C than at 5°C. Cook et al. (1966), using samples of unfrozen and pre-rigor frozen ovine semitendinosus muscle, found that variations in temperature caused muscle fibers to be in various states of contraction.

Carcass position has been found to have a definite effect on the contractile state of the muscle (Herring et al. 1965b). By vertically suspending the carcass, certain muscles are in a stretched state, as indicated by sarcomere length, while other muscles are in a shortened state.

Postmortem morphology of the muscle fiber has been shown to vary considerably, and again should be considered in many tenderness studies. Paul et al. (1944) reported that the structural appearance of the muscle fiber varied with aging. Harrison et al. (1949) found that freshly killed beef muscle showed poorly differentiated fibers that were straight to slightly wavy. After one day of storage at 1.7°C the fibers and cross striations were more distinct and the longitudinal striations less distinct. Contracture nodes, kinks and waves increased after four to nine days of storage. Disappearance of cross striae in small infrequent areas of the fibers were noted on the second day of storage, and this disintegration increased in frequency and extent as storage time increased.

Reddy et al. (1967), studying the effect of pre-rigor excision of three bovine muscles on fiber diameter and amount of fiber distortion, reported some interesting relationships in condition. The amount of fiber distortion, termed percent kinkiness, was found to be greater for the pre-rigor excised longissimus dorsi muscle than the post-rigor excised muscle. However, the opposite was found true for the gluteus medius muscle, thus supporting the postulation of Locker (1960) and the results of Herring et al. (1965b) that different internal strains among muscles exist in the vertically suspended carcass. These workers also found that the diameter of the fibers of the pre-rigor excised semitendinosus muscle was greater than the fiber diameter of the post-rigor excised muscle,

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Table 1—The influence of tension during chilling on pre-rigor excised bovine muscle.

		Semitendinosus				Semimembranosus				
Tension[1]		0	1,000	2,500	5,000	0	1,000	2,500	5,000	SE[2]
Fiber diameter[3]	μ	67.05	58.88	54.41	57.56	62.85	54.29	52.98	52.43	1.28
Sarcomere length[4]	μ	1.94	2.34	2.60	2.88	1.91	2.45	2.73	2.82	0.08
Kinkiness[5]	%	32.86	22.30	25.90	33.40	36.57	21.80	15.80	13.83	3.71
Shear force[6]	lb	33.55	26.12	26.39	26.79	29.25	26.68	24.38	25.12	2.06

[1] Grams of pull using a tensiometer.
[2] Standard error.
[3] (ST) 67.05 > 58.88 ($P < 0.01$).
(SM) 62.85 > 54.29 ($P > 0.01$).
[4] (ST) 2.88 > 2.34 > 1.94 ($P < 0.01$).
2.88 > 2.60 > 2.34 ($P < 0.05$).
(SM) 2.82 > 2.45 > 1.91 ($P < 0.01$).
2.73 > 2.45 ($P < 0.05$).
[5] (SM) 36.57 > 21.80 ($P < 0.01$).
[6] (ST) 33.55 > 26.79 ($P < 0.05$).

which was apparently due to the shortening of the muscle.

This study was designed to elucidate the relationship of muscle tension to fiber diameter, sarcomere length, and the amount of fiber distortion resulting from various pre-rigor levels of tension. The relationship of the previously mentioned structural characteristics to tenderness were also studied.

EXPERIMENTAL

FIVE STEERS ranging in weight from 433 to 503 kg of comparable finish and grade were sacrificed in accordance with the practices normally employed in the laboratory and by industry (Wellington, 1953). The semitendinosus and semimembranosus muscles were excised from both hindquarters approximately 45 min postmortem, trimmed of excess external fat, and divided into two samples each of approximately 6x6x21 cm (four samples per muscle). These samples were then subjected to four levels of tension (0, 1,000, 2,500, 5,000 g) by the use of a tensiometer and held in this state at approximately 1.1°C for 48 hr. Each sample was covered with a fibrous bologna casing to prevent excessive moisture loss. The samples were then removed from the tensiometer, histological core samples taken, and the remainder wrapped and frozen for shear determinations.

The samples used for structural characterization consisted of removing two (0.63x1.27 cm) cores from each end of the muscle section. These were put into a 10% buffered formalin fixative for approximately one week and held at approximately 1.1°C.

A sample of tissue was removed from each fixed core and blended in a slow speed Waring Blendor, blades reversed, for one minute in a 10% formalin media to dislodge the fibers. The suspension of fibers, in the formalin media, was observed in a 5 cm petri dish under a regular light microscope at 150× magnification. Twenty-five fibers were measured per section giving a total of 100 fibers per sample. (400 measurements per muscle). All fibers, broken and distorted, were measured at their widest diameter, and observed in the field of a predetermined course.

Percent kinkiness, characteristics of the fiber in rigor, was determined at the same time as the fiber diameter was measured. For each fiber diameter measured a value was subjectively assigned to the condition of that fiber. The number of fibers falling into the seven classifications were recorded and multiplied by an assigned value. The products were then added and divided by the total possible value, which permitted the degree of fiber kinking and distortion to be expressed on a percentage basis.

Measuring sarcomere lengths consisted of dislodging the myofibrils, which was accomplished by blending the sample for 1 hr in a formalin media. A drop of the suspended myofibrils was then placed on a clean glass slide and held in place with a glass coverslip. No stain or mounting media was used. The slide was viewed under a phase-contrast microscope equipped with an ocular micrometer, calibrated as one micron per division, at a power of 1600×. The field course consisted of making three passes across the slide being careful not to allow fields to overlap. Sarcomere lengths were determined by collectively measuring 10 sarcomeres, on each of 25 myofibrils and calculating an average. Twenty-five myofibrils were measured per sample, or 100 measurements per muscle.

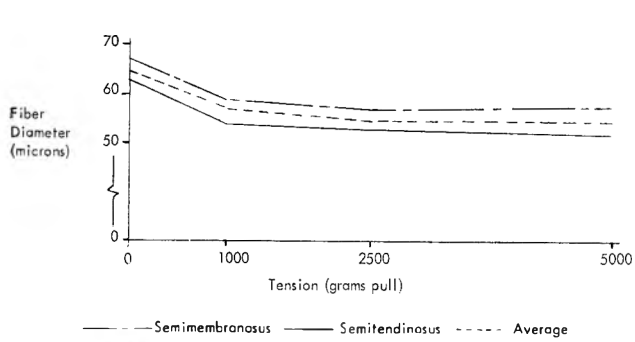
Shear values were conducted using the Warner-Bratzler shear machine. Previously frozen muscle samples were allowed to thaw at approximately 3.9°C for 24 hrs. They were then tagged and cooked in oil preheated to 135°C. Each steak was allowed to attain an internal temperature of approximately 68.3°C, as indicated by a conventional meat thermometer placed in the center of the sample. The cooked steaks were then placed in the 3.9°C cooler, covered, and allowed to equilibrate to an internal temperature of 3.9°C for approximately 24 hr. Cores were removed from the center of the muscle sample by the use of a mechanical coring device mounted on a drill press. Six shear determinations were made on each 2.54 cm core. A total of 24 shear determinations were made per muscle.

All data were analyzed by analysis of variances and Duncan's new multiple range test.

RESULTS & DISCUSSION

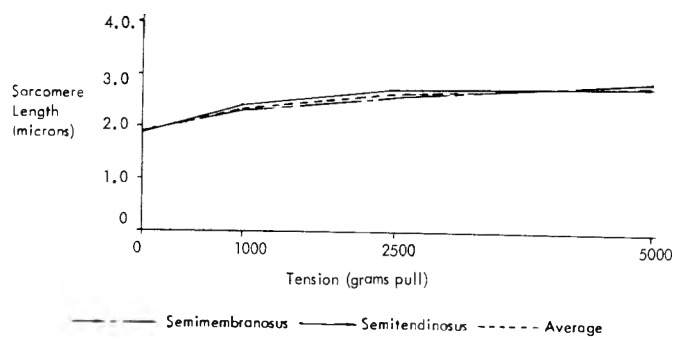
VARIATION ON FIBER diameter was found to be highly significant ($P < .01$) for the four tension treatments. However, highly significant differences ($P < .01$) were also noted for muscles and animals. This was to be expected and is in agreement with previous research. The average fiber size for each level of tension is shown in Table 1.

Further analysis, using Duncan's new multiple range test, indicated that there was a highly significant decrease



Semimembranosus and Semitendinosus are significant at P<.01 for ranges of 0-1000, 0-2500, and 0-5000.

Fig. 1—The effect of tension on fiber diameter.



All ranges significant at P<.01 for both muscles with the exception of the range 2500-5000 which was significant at P<.05.

Fig. 2—The effect of tension on sarcomere length.

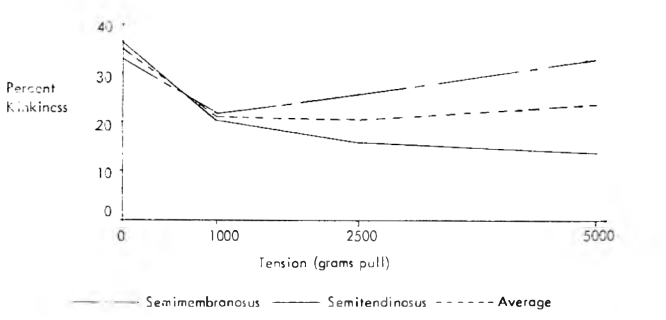


Fig. 3—*The effect of tension on percent kinkiness.*

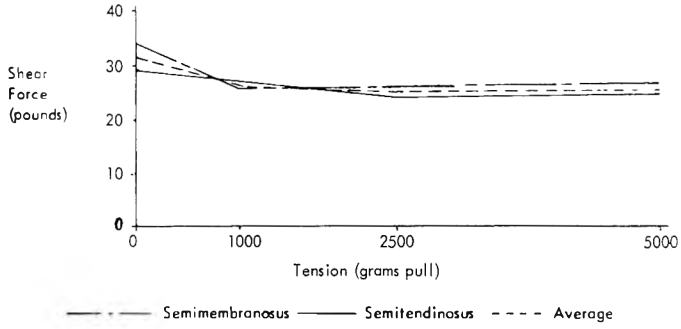


Fig. 4—*The effect of tension on shear force.*

($P < 0.01$) in fiber diameter with increasing tension to the 1,000-g pull treatment, then a gradual leveling off with succeeding increases in tension. These changes are graphically presented in Figure 1. It would seem logical that as the individual muscle fiber was stretched there was a corresponding decrease in fiber diameter. The muscle fiber evidently approached a point of physical limitation with respect to stretching at the 1,000-g pull treatment, with only a slight decrease in diameter to the 2,500-g pull.

The semimembranosus muscle had consistently smaller fiber diameters at each degree of tension than the semitendinosus muscle, thus indicating true inherent differences between the two muscles.

The analysis clearly indicated that with succeeding increases in tension above 1,000 g, a corresponding increase in sarcomere length occurred (Table 1). A graphic view of the data are presented in Figure 2. Duncan's test indicated that all ranges were highly significant ($P < 0.01$) except the ranges 1,000–2,500 and 2,500–5,000, which were significant at ($P < 0.05$). These findings are in general agreement with the work done by Herring et al. (1965a) who noted that sarcomere length of the semitendinosus muscle shortened as a result of pre-rigor excision, but that the pre-rigor excised stretch-restained semitendinosus muscles generally exhibited longer sarcomeres than the control samples.

Variation in percent kinkiness was found to be highly significant ($P < 0.01$) for the different degrees of tension. Significant differences ($P < 0.05$) were noted between muscles and for a muscle by tension interaction (Fig. 3). The differences between muscles and the significant interaction can be accounted for by differences in the physical properties of the two muscles. Herring et al. (1965a) reported that the semitendinosus muscle was able to retain initial length after being stretched. whereas the psoas major was not. Their data along with the findings in this investigation confirm differences in the physical properties of the two muscles. In this experiment, there was some difference in connective tissue content of the muscles (Lee et al. 1967). which feasibly could have had an effect on the amount of fiber kinking and distortion.

As can be seen in Table 1. both muscles descreased in percent kinkiness to the 1,000-g pull treatment. The semimembranosus muscle tended to decrease and the semitendinosus muscle tended to increase in percent kinkiness with increasing amounts of tension. This is in agreement with the findings of Reddy et al. (1967) who noted that the pre-rigor excised longissimus dorsi muscle had a higher percent kinkiness than the post-rigor excised muscle, indicating that in the post-rigor excised muscle the strains induced by vertical suspension of the carcass does inhibit kinking of the fibers to a degree.

Shear force was found to differ significantly ($P < 0.05$) for the tension treatments. Significant differences ($P < 0.05$) were also noted between animals. Shear force is a highly variable measure and it is difficult to pick up small differences without many shear determinations. A more precise measurement would have been desirable for comparing relationship in this study.

When the data for both muscles were combined and analyzed there was a significant decrease ($P < 0.05$) in shear force between zero and the 1,000-g pull treatment, then it leveled off (Fig. 4). This type of response was also characteristic of several of the previously mentioned variables, thus indicating that a true relationship of these variables may exist.

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Enzymic and Acid Hydrolysis of Sucrose as Influenced by Freezing

SUMMARY—*Sucrose hydrolysis catalyzed by invertase was studied over the temperature range 12 to −22°C in solutions of various concentrations. Freezing resulted in a marked decrease in invertase activity. Factors considered as having a possible role in the inhibition of invertase by freezing were: (1) the presence of ice crystals, (2) the increased concentration of solutes resulting from ice formation, and (3) a temperature effect in excess of that predicted from the Arrhenius equation (secondary temperature effect). It was shown that ice crystals formed at the rate used in this study had no effect on invertase activity, and that the decrease in invertase activity at sub-zero temperatures was quantitatively accounted for by the concentration effect and the secondary temperature effect. High levels of sucrose and buffer (ionic strength) were found to inhibit invertase activity. Sucrose hydrolysis catalyzed by acid (HCl) was studied over the temperature range 12 to −16.5°C, and an Arrhenius plot of the data was linear between 12 and −7°C. Once the solution was frozen (below −6.8°C), the effect of temperature on the reaction rate constant no longer obeyed the Arrhenius equation, and the reaction rate constant was found to exhibit a maximum between −8.5 and −16.5°C. The rate of sucrose hydrolysis catalyzed by acid was influenced by cooling and conventional freezing in a manner quite different from that observed with invertase-catalyzed hydrolysis.*

INTRODUCTION

ENZYMIC ACTIVITY in frozen foods is a concern, particularly in those not heated prior to freezing. The activity of several enzymes has been studied in frozen aqueous systems, among them invertase by Kertesz (1942) and Sizer et al. (1942), lipase by Balls et al. (1938) and Kiermeier (1948), catalase by Kiermeier (1948), proteinases by Mergentime et al. (1946), chymotrypsin and cathepsin by Lineweaver (1939) and peroxidase by Tappel (1966). In general, enzymes are less active in frozen systems than in liquid solutions at the same temperature, although exceptions to this rule have been reported (Kiermeier, 1948).

Sizer et al. (1942) studied trypsin, lipase and invertase activity in the temperature range 50 to −70°C and reported sharp breaks in the Arrhenius plots near the freezing point of each system. For invertase they reported activation energies (μ) of 11,100 cal/mole above −2°C. and 60,000 cal/mole below −2°C. These results prompted Sizer (1943) to suggest that freezing produced a change in enzyme action.

Joslyn (1951) reviewed the effects of concentration and freezing on enzymes and suggested that increased concentration of solutes and changes in physical and colloidal properties that occur during solidification may be responsible for reduced enzymic activity in frozen systems.

The effect of sub-zero temperatures on phosphatase- and peroxidase-catalyzed reactions in unfrozen systems was investigated by Maier et al. (1955). Resulting Arrhenius plots were linear from 20 to near 0°C and curvilinear from 0 to −30°C. Enzymic reaction rate constants were more temperature dependent below zero than above. These results, according to the authors, are in agreement with a theory proposed by Kavanau (1950) to the effect that: (1) enzymes exist in reversible equilibrium between active and inactive forms and (2) low temperatures are especially effective in altering this equilibrium, thereby causing nonlinear Arrhenius plots. According to Kavanau, lowering the temperature increases enzymic intramolecular hydrogen bonding, alters the specific configuration of the enzyme and masks active sites.

Hultin (1955) further developed the thermodynamics involved in the Kavanau theory and used the data of Sizer et al. (1942) on trypsin, lipase and invertase to calculate values for: (1) ΔH^*, the enthalpy of activation ($\mu - RT$), (2) ΔH, the heat of reversible denaturation between active and inactive forms of the enzyme, and (3) $T_{1/2}$, the temperature at which half of the total enzyme is in the active form ($K_{equil} = 1$). For invertase the following values were obtained: $\Delta H^* =$ 10,000 cal/mole, $\Delta H = -51{,}000$ cal/mole and $T_{1/2} = 269.6°K$ (−3.4°C).

In making his calculations Hultin assumed that the sucrose concentration was optimum for invertase activity is markedly dependent on sucrose concentration (Nelson et al. 1928) and since the data of Sizer et al. (1942) included rate values from partially frozen samples (thereby altering the sucrose concentration in the unfrozen phase), Hultin's assumptions do not appear valid. The present study was done to help clarify why invertase inhibition occurs during freezing.

The influence of freezing on nonenzymic chemical reactions has recently received considerable attention, and the effects are strikingly different from those observed with enzyme-catalyzed reactions. It has been shown, for example, that numerous bimolecular reactions proceed more rapidly in the presence of ice than in its absence (Grant et al., 1961; Prusoff, 1963; Butler et al., 1964; Weatherburn et al., 1964; Alburn et al., 1965; Grant et al., 1965a,b, 1967; Kiovsky et al., 1966; Pincock et al., 1966). Mechanisms suggested as contributing to accelerated reaction rates in the presence of ice are: (1) a more favorable orientation of reactants, (2) enhanced proton mobility in systems dependent on proton transfer, (3) concentration of reactants in unfrozen regions, (4) nucleophile association, and (5) catalysis by ice.

Pincock et al. (1966) developed an equation relating the rate of reaction in a frozen sample to the concentration of solutes in the unfrozen solution. According to this equation, a maximum reaction rate constant should occur at a subfreezing temperature. Fairly good agreement was in fact obtained between calculated and observed rate constants for glucose mutarotation in frozen aqueous solutions (Kiovsky et al., 1966). In opposition to this view, Grant et al. (1967) observed that changes in the reaction rate upon freezing cannot always be explained on the basis of concentration changes and that a mechanism such as catalysis by ice may be operative.

Rates of acid-catalyzed sucrose hydrolysis in frozen and unfrozen systems were determined in the present study so that acid and enzymic catalysis could be compared.

EXPERIMENTAL

Materials

The invertase concentrate was prepared by dissolving 0.8 g of dry invertase (Yeast Invertase, Grade B, CalBiochem, Los Angeles) in 129.2 ml of 95.1% glycerol (Baker Co., A.C.S. Standard Grade, 95.1% [w/v]) and diluting the solution to 200 ml with 0.02*M* sodium citrate buffer (pH 4.45). (All chemicals unless otherwise specified were A.R. or A.C.S. purity. Throughout this report the quantity of invertase used will be expressed in "units," where one unit of enzyme is defined as the amount that catalyzes the trans-

formation of one micromole of substrate per min under defined conditions [Anonymous, 1965]). For the invertase used here, it was determined that 0.5 ml of a 1/10 dilution of enzyme concentrate catalyzing the hydrolysis of 24.5 ml of a 2.5% (w/v) sucrose solution at pH 4.62 and 6°C contained 5.62 units of invertase.

Methods

Five solutions of different water content but of the same kind and ratio of solutes were used to determine the effect of temperature, concentration and ice crystals on the activity of invertase. The compositions of these five solutions are shown in Table 1, and they are numbered in accordance with their concentrations. For example, the 10x sample contains the same amount of solutes as the 1x sample, but in 1/10 the total volume.

The effect of temperature on invertase activity was determined by using the 15x solution. This solution enabled temperature effects to be determined down to −16.5°C (freezing point of the 15x solution) without interference from a change of state. The second effect, that of concentration (water content), was determined by comparing the reaction rate constants of the 1x, 5x, 10x and 15x samples at temperatures from 0 to 12°C. The third effect, that of ice crystal presence, was determined by measuring the reaction rate constants of the various unfrozen concentrated samples at their respective freezing points and comparing these values to the reaction rate constants of the frozen 1x samples at comparable temperatures.

The validity of this method rests on two assumptions: (1) that the manner of freezing had no influence on the activity of invertase, and (2) that an unfrozen concentrated solution at its freezing point and the frozen 1x sample at the same temperature had fluid phases of identical composition (% basis). The first assumption was found valid by actual test. The second assumption appeared reasonable since: (1) all solutions were prepared so that they differed only in water content and (2) the composition of the unfrozen phase of a partially frozen ideal solution depends only on the temperature (Mazur, 1966). It therefore follows that an unfrozen sample at its freezing point, and the partially frozen 1x sample at the same temperature, should exhibit the same invertase activity (per ml of unfrozen material) unless the presence of ice crystals exerts an effect.

The freezing points of the various solutions (1x, 3x, 5x, 10x and 15x) were determined in triplicate by a thawing curve technique, and the following values were obtained (concentration factor, °C): 1x, −0.6 ± 0.2; 3x, −1.9 ± 0.2; 5x, −3.7 ± 0.2; 10x, −8.5 ± 0.2; and 15x, −16.5 ± 0.4. All freezing point measurements were made in the absence of invertase to avoid possible errors due to sucrose hydrolysis. Considering the molecular weight of the enzyme and the small amount normally used, its absence would have no significant influence on the freezing point values as reported.

Determination of reaction rate constants in enzyme solutions. Reaction rate constants were determined in duplicate for the unfrozen solutions shown in Table 1. The samples were equilibrated for 20 min at the desired reaction temperature, invertase was added and aliquots were periodically removed and analyzed until at least 25% of the sucrose had been hydrolyzed. This degree of hydrolysis is regarded as necessary for determination of first order reaction rate constants for invertase-catalyzed sucrose hydrolysis (Neuberg et al., 1950). The amount of reducing sugar was determined by the colorimetric procedure of Somogyi, (Somogyi, 1952; Nelson, 1944). First order reaction rate constants (slope of the line obtained from a plot of ln mg unhydrolyzed sucrose versus reaction time) were calculated by linear regression analysis.

Each reaction rate constant for a frozen 1x sample at a given subfreezing temperature was derived by reacting no fewer than 10 identical 1x samples (Table 1) for various times. All constituents except the enzyme were equilibrated at 0°C, following which the enzyme was added and each sample was shell-frozen by rotating the vessel in a dry ice-acetone bath (200 rpm for 90 sec). The samples were transferred to a bath at the desired sub-zero reaction temperature and allowed to stand for 20 min to attain temperature equilibrium (duration determined experimentally). Two samples were immediately removed (regarded as zero time), thawed in hot water (completely thawed in ca. 90 sec) and analyzed. Additional duplicate samples were removed periodically, and the reaction rate constant was calculated by regression analysis of the data.

Although no measurement was made of the amount of sucrose hydrolysis that occurred during thawing, the amount was undoubtedly small because of the short time involved, and probably quite constant (within a given series of samples being used for a single determination of k) judging from the good linearity achieved for each plot of ln mg of unhydrolyzed sucrose versus reaction time. It is therefore highly unlikely that the thawing procedure had a significant effect on the observed k values.

Table 1—Composition of invertase solutions containing the same kind and ratio of solutes but different amounts of water.

Sample number (concentration factor)	Total solutes[1] (g)	Ratio of enzyme/sucrose/buffer[2] (units/mg/ml)	Sample volume[3] (ml)
1x	0.8695	5.62/612.5/0.78	24.5
3x	0.8695	5.62/612.5/0.78	8.16
5x	0.8695	5.62/612.5/0.78	4.90
10x	0.8695	5.62/612.5/0.78	2.45
15x	0.8695	5.62/612.5/0.78	1.63

[1] The total solutes consisted of 0.6125 g sucrose, 0.164 g sodium acetate, 0.120 g acetic acid, and 0.02 mg (5.62 units) invertase. All samples had pH values of 4.62 ± 0.02.

[2] The volume of sodium acetate buffer expressed in this ratio represents the ml of 7.65 ionic strength buffer contained in the total volume shown in column 4.

[3] Actually a 24.5 ml volume of the appropriate composition was always utilized to avoid large errors associated with small volumes. The results were then divided by the appropriate concentration factor (sample number, column 1) to bring them into accord with the indicated volumes.

Acid-catalyzed hydrolysis of sucrose. Observations of acid-catalyzed sucrose hydrolysis in the temperature range 12 to −16.5°C were included in this study, since such data is apparently unavailable in the literature and it is of interest for comparative purposes. It was considered desirable to work at an acid concentration similar to that found in fruits. However, preliminary studies using HCl concentrations up to 0.189*N* (p*H* 0.7) showed that the reaction proceeded so slowly that changes could not be measured accurately over an 8-hr period.

A higher HCl concentration of 1.48*N* was therefore resorted to, enabling the reaction rate constant at 6°C to be determined accurately in a 4-hr period. Samples for the acid-catalyzed studies each consisted of 20 ml of 1.77*N* HCl and 4 ml of 15.31% (w/v) sucrose (612.5 mg dry sucrose), with the final solution being 1.48*N* in HCl. The freezing point of this solution was −6.8 ± 0.2°C as determined from the thawing curves of three replicates. First-order reaction rate constants were determined using procedures described earlier.

RESULTS & DISCUSSION

Enzyme-catalyzed hydrolysis of sucrose

Arrhenius plots (log k versus 1/T) of invertase-catalyzed sucrose hydrolysis over the temperature range 12 to −22°C are shown in Figure 1. The general shape of the Arrhenius plot for the 1x sample over this temperature range is in general agreement with the findings of Sizer et al.

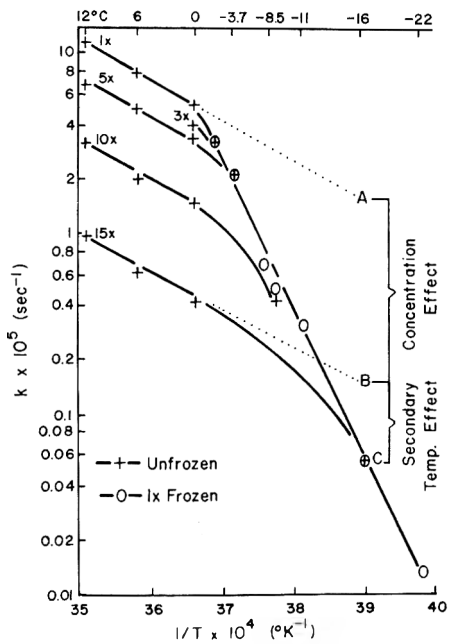


Fig. 1—Influence of cooling and freezing on the first-order reaction rate constant for hydrolysis of sucrose catalyzed by invertase.

(1942). There is a marked increase in slope negativity at a temperature just below the freezing point of the 1x sample (−0.6°C), and according to Sizer (1943) this indicates a change in enzyme mechanism. The likely causes of the change in slope will be discussed later.

The activation energy for invertase, as calculated from the slope of the 1x Arrhenius plot between 12 and 0°C, was found to be 10,100 cal/mole. This agrees fairly well with the value of 11,100 cal/mole reported by Sizer et al. (1942). The calculated activation energy of 39,100 cal/mole for the 1x sample at temperatures below freezing was considerably less than the 60,000 cal/mole reported by Sizer et al. It is difficult to judge the seriousness of this discrepancy since their reported activation energy was the only one found for comparison, and since their conditions were somewhat different than the conditions employed in this study.

Calculated activation energies for the 5x, 10x and 15x solutions over the temperature range 12 to 0°C were also 10,100 cal/mole, indicating that the activation energy is independent of concentration over the range studied.

Unfrozen concentrated samples, when cooled below 0°C, exhibited reaction rate constants less than would be predicted from the Arrhenius equation. This decline in the rate constant (the curved portion of the Arrhenius plot for concentrated samples) must be attributed to an additional effect of temperature since no ice was present. This nonlinearity of log k versus 1/T is in accord with Kavanau's theory (1950) that active and inactive forms of the enzyme exist in a reversible equilibrium especially sensitive to low temperatures. Accordingly, low temperatures affect the reaction rate constant to a greater extent than predicted by the Arrhenius equation, and the plot becomes nonlinear. Decreasing the temperature to sub-zero values supposedly results in increased intramolecular hydrogen bonding, thereby altering the configuration of the enzyme.

The fact that ice crystals (when formed at the rates used in this study) had no effect on invertase activity can be clearly seen by comparing the reaction rate constants of the concentrated unfrozen samples, at their respective freezing points, with the reaction rate constants of the frozen 1x sample at comparable temperatures. For example, at −3.7°C (the freezing point of a 5x concentrated sample) the reaction rate constant in the frozen 1x sample was identical (within experimental error) with the reaction rate constant determined in the 5x concentrated (unfrozen) sample. Similar results were obtained at the freezing points of the 3x, 10x and 15x solutions.

It is also of interest to compare the frozen and unfrozen samples with respect to the absolute quantities of sucrose hydrolyzed per unit time. These values can be readily calculated from the k values in Figure 1 ($t_{1/2} = 0.693/k$). Consider for example, the 1x and 15x samples at 0°C and −16.5°C. To hydrolyze half the total amount of sucrose at 0°C would require 3.8 hr in the 1x sample and 48.1 hr in the 15x sample (both unfrozen). On the other hand, at −16.5°C, hydrolysis of half the total sucrose would require 363 hr in both the 1x (frozen) and the 15x (unfrozen) samples.

Constituents responsible for invertase inhibition

It is evident that at any given temperature above 0°C the reaction rate constant (k) decreases as the concentration is increased from 1x to higher values. The decrease in k must be caused by one or more of the following constituents: invertase, sucrose or buffer.

To determine the effects of invertase and sucrose concentration on k, a study was conducted in which the sucrose and enzyme concentrations were varied, but ionic strength was held constant at the 1x level (0.12 ionic strength). Two sets of solutions containing various amounts of sucrose were prepared and equilibrated at 0°C. The amount of enzyme used in the 1x sample of Figure 1 and Table 1 was added to one set of solutions, and the amount of enzyme used in the 15x sample of Figure 1 and Table 1 was added to the other set. The reaction rate constants were then determined at 0°C and the results are shown in Figure 2.

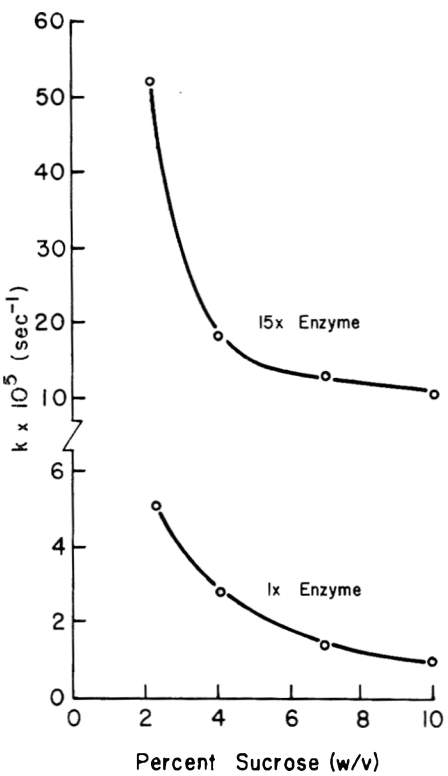


Fig. 2—Effect of invertase and sucrose concentration on invertase activity at 0°C. Compositions of the samples were: All samples were 0.12 in ionic strength and contained 2.0, 2.5, 4.0, 7.0 or 10.0% (w/v) sucrose. The 1x enzyme samples contained 5.62 units of invertase; the 15x enzyme samples contained 84.30 units of invertase.

At any given sucrose concentration (for example, 7% [w/v] sucrose), the k value for the 15x enzyme solution was always on the order of 10 times greater than the k value for the 1x enzyme solution. This indicated, as would be expected (Gutfreund, 1965), that the decreases in k, which occurred as concentration was increased (Fig. 1), cannot be attributed to invertase. However, the rate depressions observed in Figure 1 would have been less had k varied in direct proportion with enzyme concentration.

The effect of increased sucrose concentration on k is also demonstrated in Figure 2. At both enzyme concentrations, the reaction rate constant decreased as the sucrose concentration increased. This is in agreement with the results of Plunkett et al. (1951). Thus the decrease in k that occurred as concentration was increased in the above 0°C region of Figure 1 can be attributed in part to sucrose.

If the results shown in Figure 2 had been expressed as initial reaction rates (mg reducing sugar produced per unit time) rather than reaction rate constants, a maximum reaction rate would occur on both curves at approximately 7.5% (w/v) sucrose. This is in agreement with the results of Nelson et al. (1928).

The effect of ionic strength (buffer) on invertase activity was determined by comparing the reaction rate constants of two solutions differing only in ionic strength. A 15x solution was prepared like that listed in Table 1 except that its ionic strength was equal to that of a 1x solution (0.12 ionic strength). A control 15x solution was prepared in exact accordance with that listed in Table 1. Reactions were run at 0°C, and k values of 0.86×10^{-5} sec^{-1} and 0.40×10^{-5} sec^{-1} were obtained, respectively, for the 15x solution of 0.12 (low) ionic strength and the 15x control. Thus, it was evident that the low invertase activity of the concentrated samples of Figure 1 was caused in part by their high ionic strengths.

In summary, the reaction rate constant for invertase-catalyzed sucrose hydrolysis was observed to decrease with increased solute concentration (enzyme, buffer and sucrose). This decrease in invertase activity can be attributed partly to increases in buffer concentration (ionic strength) and partly to increases in sucrose concentration. Increases in invertase concentra-

tion were found to result in increased reaction rates, but this effect was more than counterbalanced by the inhibitory effects of the buffer and sucrose.

Analysis of changes in the reaction rate constant

By examining the data in Figure 1, it is possible to determine the relative importance of concentration and secondary temperature effects (effects in addition to those predicted by the Arrhenius equation) on invertase activity.

If the linear portion of the Arrhenius plot (above 0°C in Fig. 1) is extrapolated to sub-zero temperatures, a reference line is provided to which observed sub-zero k values can be compared. This extrapolated line represents the expected results if the concentration did not change during ice formation and if there were no temperature effects other than those predicted by the Arrhenius equation (primary temperature effect). For example, point A on Figure 1 represents the extrapolated reaction rate constant for a 1x solution at −16.5°C (freezing point of a 15x solution).

Point C on Figure 1 represents the experimentally determined reaction rate constant in a frozen 1x solution at −16.5°C. The difference between the extrapolated 1x k value (point A, Fig. 1) and the experimentally determined 1x k value (point C, Fig. 1) represents the decrease in k due to: (1) the effects of increased concentration from 1x to 15x, and (2) secondary temperature effects.

To separate concentration effects from secondary temperature effects, an extrapolation must be made of the linear portion of the Arrhenius plot (Fig. 1) for the concentrated solution in question (15x in this instance). Point B on Figure 1 represents the extrapolated reaction rate constant for the 15x solution at −16.5°C, and distance AB represents the decrease in k due to increasing the concentration from 1x to 15x. Of the total decrease in k (line AC) distance AB or fraction AB/AC is caused by concentration effects, and distance BC or fraction BC/AC is caused by secondary temperature effects.

Calculations of the type just described were carried out for each of the concentrated solutions and the results appear in Table 2.

It is apparent that the reaction rate constants of frozen 1x solutions at various sub-zero temperatures are influenced more by concentration changes than by secondary temperature effects. There also appears to be a tendency for the concentration effect to become more important as the temperature is decreased. However, this trend must change at some temperature below −16.5°C since a point will be reached eventually where a decline in temperature will result in little, if any, change in concentration. The fact that 93% of the water is frozen at −16.5°C (column 3, Table 2) indicates that solidification and the concentration process are not far from completion at this temperature.

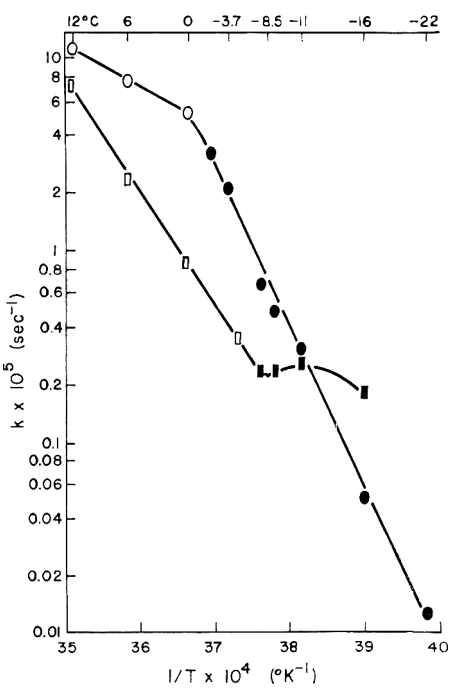
Table 2—Influence of concentration changes and secondary temperature effects[1] on invertase activity at various sub-zero temperatures.

Concentration[2]	Temperature (°C)	% H_2O solidified[3]	Fraction of total sub-zero decrease in k[4] caused by: Concentration effect	Secondary temperature effect
1x	0	0	0	0
3x	−1.9	67	0.83	0.17
5x	−3.7	80	0.78	0.22
10x	−8.5	90	0.90	0.10
15x	−16.5	93	0.95	0.05

[1] Temperature effects in excess of those predicted from the Arrhenius equation.
[2] Compositions of the samples are shown in Table 1.
[3] These values were obtained by calculation. % H_2O solidified = [1.0 − (1/concentration factor)]100.
[4] Reference point is the k value for the 1x sample as obtained by extrapolating the above zero line to sub-zero values (see Fig. 1).

Acid-catalyzed hydrolysis of sucrose

The effects of temperature and change of state on the reaction rate constants for hydrolysis of sucrose catalyzed by invertase and by acid are shown as Arrhenius plots in Figure 3. The data presented for enzymic hydrolysis are the same as that shown for the 1x sample in Figure 1.



○ *catalyzed by invertase, unfrozen samples.*
● *catalyzed by invertase, frozen samples.*
□ *catalyzed by acid, unfrozen samples.*
■ *catalyzed by acid, frozen samples.*

Fig. 3—Influence of cooling and freezing on the first-order reaction rate constant for hydrolysis of sucrose catalyzed by invertase or acid.

For acid hydrolysis, a linear Arrhenius plot was obtained over the temperature range 12 to −7°C, and the activation energy was calculated to be 26,000 cal/mole. This is identical to the value reported by Sizer (1943).

Once the acid-catalyzed solution was frozen (below −6.8°C), the effect of temperature on the reaction rate constant no longer conformed to the Arrhenius equation and, in fact, k went through a subfreezing maximum between −8.5 and −16.5°C. This type of behavior has been reported for a number of bimolecular reactions in frozen aqueous systems (Grant et al., 1961; Prusoff, 1963; Butler et al., 1964; Weatherburn et al., 1964; Alburn, et al., 1965; Grant et al., 1965a, b, 1967; Kiovsky et al., 1966; Pincock et al. 1966). Mechanisms suggested as contributing to accelerated reaction rates in frozen systems were cited in the introduction.

Pincock et al. (1966) developed an equation relating the reaction rate in a frozen solution to the concentration of solutes in the thawed solution (initial concentration). Experimental data on the rate of glucose mutarotation in partially frozen aqueous solutions indicated that the concentration effect accounted for nearly all of the rate enhancement observed during freezing (Kiovsky et al., 1966). In accordance with this relationship, the reaction rate constant of an initially dilute sample (on the order of 0.05*M*) exhibited a greater increase during freezing than the reaction rate constant of a sample that was initially more concentrated. Since the sample used in this study was initially quite concentrated, the increase in reaction rate constant observed during freezing is likely smaller than would have occurred if the initial sample had been more dilute.

The fact that freezing can enhance nonenzymic bimolecular reactions, such as acid-catalyzed hydrolysis of sucrose, may be of concern in practical situations. This is particularly true of thawing since: (1) thawing of food tissue is inherently slower than freezing, and (2) the product spends most of the thawing period at a temperature very near its freezing point (Fennema et al., 1964). The last point is especially significant since the product will be in the temperature zone where enhanced rates of nonenzymic reactions are observed. As is apparent from Figure 3, the reaction rate constant for sucrose hydrolysis catalyzed by invertase follows a different and less detrimental pattern of change than that of the acid-catalyzed samples.

Judging from these results, it would seem desirable that reactions that contribute significantly to the deterioration of food during frozen storage should be investigated at temperatures just below the freezing point of the system as well as at lower storage temperatures. The information gained would help determine an optimum thawing technique.

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High Resolution Vapor Analysis for Fruit Variety and Fruit Product Comparisons

SUMMARY—By using a system incorporating large-bore open-tubular gas chromatographic columns, high resolution vapor analyses of five fresh apple products were run. The improved peak separation attainable with such a system permits more detailed comparisons between different samples. This is a considerable aid in varietal studies and in following loss and change of volatiles during processing. The apple varieties examined appear to differ mostly in the relative proportions of individual components rather than in the presence or absence of certain constituents.

INTRODUCTION

VAPOR OR HEADSPACE analysis (MacKay, 1960; Teranishi et al., 1962), offers several advantages to the investigator interested in the volatiles makeup of a food sample. The technique is relatively fast, for little sample preparation is required other than a period of equilibration at some standard temperature. Because no extraction, distillation or other preliminary separation and concentration steps are involved, the likelihood of artifact introduction or sample change is greatly minimized. Only the sample's more volatile components are examined in approximately the same proportions as they are found in the food sample's aroma. If we assume the response of the

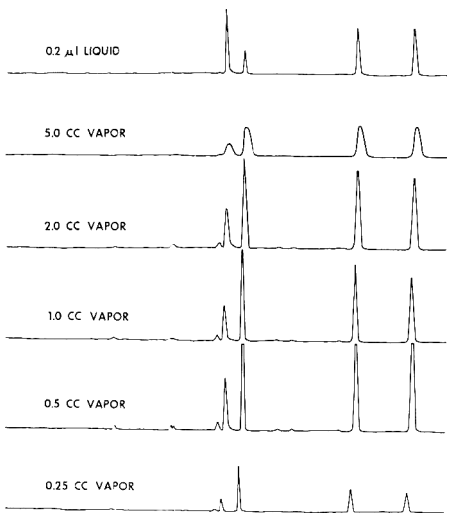

Fig. 1—Effect of sample volume on open-tubular column efficiency — 0.03 in. ID column.

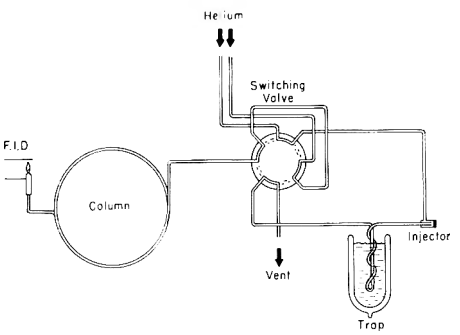

Fig. 2—Trapping system for open-tubular column vapor analysis.

flame ionization detector is linear for all compounds present, then the areas under the chromatogram peaks should indicate the relative amounts of the individual volatiles in the sample's aroma.

There are several difficulties in applying the technique. The tremendous sensitivity of the human nose to different compounds, compared with a flame ionization detector, can be a considerable problem in using gas chromatography for flavor and aroma work. An organoleptically important component may be present in the vapor above a sample in a proportion so small that it is not detected during vapor analysis. But because of a low threshold, this component can still make a significant contribution to the sample's aroma impression on the human nose.

The physical structure of the sample influences vapor-analytical results. In a whole fruit, for example, some volatiles may be contained within elements such as oil sacs or seeds, and so may appear at lower concentrations in the headspace analysis of a whole fruit than they do in vapor analysis after crushing. On the other hand, enzymatic reactions may change the volatiles makeup when sample tissue is cut or macerated (Drawert et al., 1966). In practice, the particular application will determine whether a sample should be pulverized, sliced, pressed, or left whole for vapor sampling.

Considerable care must be taken in using vapor analytical results for estimating the relative concentrations of volatile compounds *within* a food sample. Very few, if any, samples can be dealt with as ideal homogeneous solutions, with the equilibrated vapor above the sample constituted according to Raoult's Law. Nawar (1966) and Wientjes (1968) have described the effect of food sample makeup on the headspace composition above the sample.

Identification of compounds by high resolution vapor analysis faces the same problem encountered in any GC analysis of complex mixtures—separation on a single column provides very little, if any, information about identities of individual components. If identifications are required, supplementary information is usually necessary, derived from relative retention times on several stationary phases, from mass spectrometer-GC data, or most reliably, from isolation and identification of individual volatile components of the food.

In spite of these difficulties, the advantages of vapor analysis recommend it

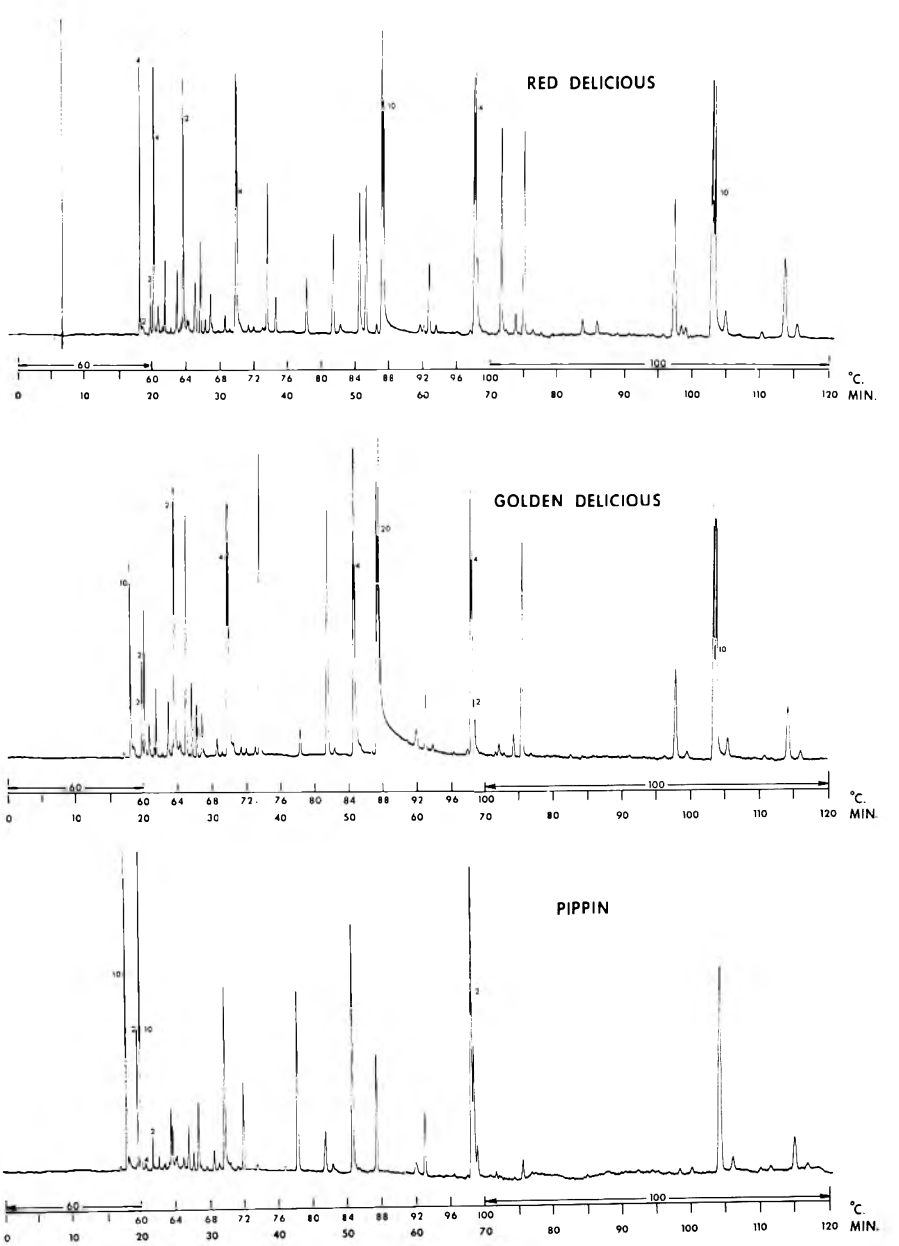

Fig. 3—Fresh apple varieties: a) Delicious, b) Golden Delicious, c) Pippin.

as a useful tool for the food researcher concerned with a food sample's aroma makeup (McCarthy et al., 1963; Kepner et al., 1964; Heins et al., 1966; Romani et al., 1966).

Most vapor analyses are conducted with packed columns of various kinds. In the larger diameters (¼ in. or greater) they can tolerate appreciable sample volumes without peak-broadening. However, packed-column separations of complex aroma mixtures are often unsatisfactory because of poor component resolution. A study was therefore initiated to see whether 0.03 in. ID open-tubular columns with their much higher efficiencies might improve separations for examination of fruit aroma composition.

A 0.03 in. ID column could not tolerate a 5–20 cc vapor sample without appreciable peak-broadening. Figure 1 shows the results obtained when increasing vapor sample volumes were injected into a 1000 ft x 0.03 in. ID column coated with methyl silicone oil. Unacceptable peak-widening appeared above 0.5 cc. Unfortunately, a 0.5 cc sample usually contains insufficient material for satisfactory analysis, Fig. 1.

A pre-column trapping system (Fig. 2) for preliminary concentration of condensible volatiles from the vapor samples was then tried. An adaptation of cryogenic gas chromatography, in which the column itself is initially cooled to act as a trap, is an alternative approach to the sample size problems encountered with open-tubular columns (McEwen, 1964; Rushneck, 1965; Heins et al., 1966). For our purposes, a separate larger ID trap [2 in. of 0.075 in ID, and 4 in. of 0.04 in. ID tubing coated with methyl silicone oil SF 96(50)] seemed preferable.

Separations subsequently obtained on a temperature-programmed 0.03 in. ID column were a considerable improvement over earlier packed column separations. The results could also be directly correlated with separations of liquid essence concentrates obtained on 500 ft × 0.02 in. ID open-tubular columns. Compound identifications made in this discussion are based upon previous mass-spec-GC work conducted with these 0.02 in. columns. Some typical vapor chromatograms obtained by this method are shown in Figs. 3–5.

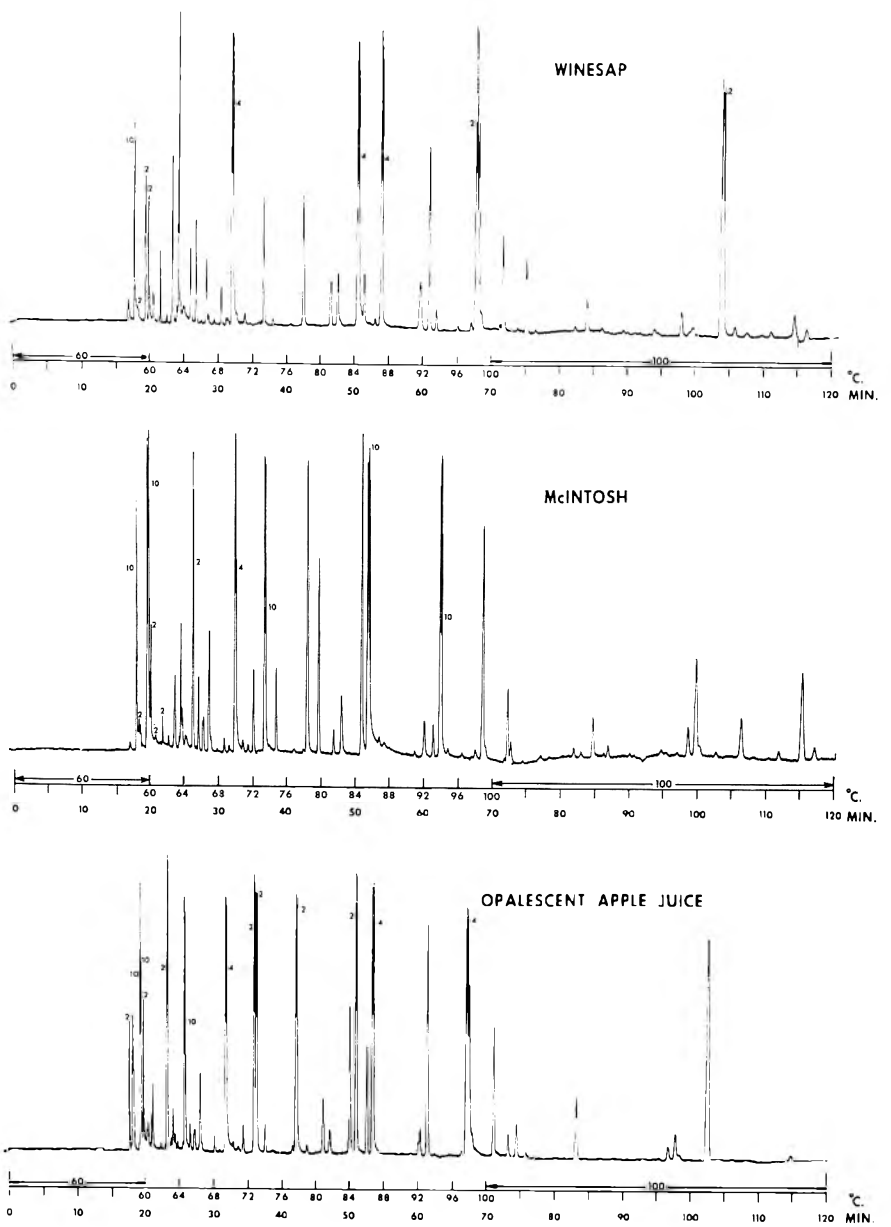

Fig. 4—Fresh apple varieties and apple product: a) Winesap, b)McIntosh, c) Opalescent apple juice.

EXPERIMENTAL

THE GAS chromatograph, dual flame ionization detector-electrometer assembly, and trapping system were constructed of two 900 ft × 0.03 in. ID × 0.062 in. OD Type 316 stainless steel open-tubular columns (Handy & Harman Tube Co., Inc.) coated with a mixture of 95% SF 96(50) (methyl silicone) and 5% Igepal CO-880 [nonylphenoxypoly-(ethyleneoxy)ethanol], then conditioned at 200°C for several days with water-saturated nitrogen at 30 psi. The columns were then operated in a differential manner in the chromatograph.

To conduct a vapor analysis, the fruit sample (100 g of peeled and cored freshly-sliced apple; 100–200 g of apple product) was placed in a water-jacketed (35°C) Pyrex flask (250 ml) which was closed with an aluminum foil cap. After 15 min, the needle of a clean glass syringe was inserted through the cap and 20 cc of vapor was withdrawn without "pumping" the syringe. The sample was then injected into the trapping system (Fig. 1), with the stainless steel trap cooled in a solid carbon dioxide-acetone bath.

After the condensible material was trapped, the bath was removed, the switching valve (Carle Instruments Inc.) was rotated and the trap was heated rapidly to 130–140°C with a hot air blower. The time interval from removal of the bath to attainment of maximum temperature is 13–14 sec. No peaks were detected when blanks were run.

The fresh fruit and the fruit products were purchased in local markets. The apple slices and sauce were canned, the juice and apple butter were packaged in glass containers.

RESULTS & DISCUSSION

TABLE 1 lists retention times of chromatogram peaks which are representative of the 10 compounds listed. Assignments were determined by co-injection of vapor from authentic samples along with Red Delicious apple vapor. The compounds chosen were found in Delicious and

Gravenstein apple essences by the researchers cited above. Assignments for the other apple varieties were made by comparing the Red Delicious run with the other chromatograms. This is not a rigorous identification technique, but is sufficiently reliable for the purposes of this discussion.

Some shortening of retention times may be noted from the table. The samples were run in the order listed, so this shortening is likely due to initial aging of the newly-coated column.

Significant differences are apparent on inspection of the five chromatograms of fresh apple vapor shown in Figures 3 and 4. Not only does the total amount of volatiles vary considerably among varieties (e.g. Red Delicious vs. Pippin), but the relative concentration of individual components in a sample's vapor also appears distinctive for each variety (e.g. Red Delicious vs. McIntosh) under the experimental conditions described.

Differences in total volatiles content could be detected almost as easily with a low-resolution packed column as with a large-bore open-tubular column. However, measurement of an individual compound's relative concentration required resolution of the corresponding chromatogram peak from its neighbors, and this resolution is most easily obtained with open-tubular columns. Paillard (1967) reported a similar comparison of eight apple varieties using activated carbon-trapped liquid samples and packed GC columns.

As might be expected, any possible correlation of processed apple product vapor chromatograms (Figs. 4 and 5) with those of fresh apples depends upon the nature and extent of the processing involved. The opalescent apple juice compares quite well with the two Delicious varieties, as far as presence of corresponding peaks is concerned. This degree of correlation is not attained with canned slices and other more highly-processed products. The apple sauce (Gravenstein) and apple butter (cider added; no spices) chromatograms are typical of such thoroughly-cooked products. The total volatiles concentration is relatively low, with an apparently-greater proportion of short retention time components than in fresh fruit vapor.

At present, there is no clear explanation for this latter observation. Cooking alone might deplete low-boiling constituents more than high-boilers. Short retention time compounds might have developed in the product during storage, or aroma condensate material might have been blended into the material after processing.

The examples illustrate the potential of large-bore open-tubular columns in vapor analysis. Even without actual identification of all vapor constituents, the technique should prove useful for variety

Table 1—Time in minutes.

Compound	McIntosh	Golden Delicious	Pippin	Winesap	Red Delicious	Juice	Slices
n-Hexanal	51.0	50.8	50.7	50.7	50.7	50.2	49.9
Ethyl butyrate	52.0	51.7	51.6	51.5	51.7	51.1	50.9
1-Propyl propionate	53.5	53.4	53.3	53.2	53.2	52.6	52.5
1-Butyl acetate	54.3	54.3	54.1	54.0	54.1	53.5	53.4
trans-2-Hexenal	61.4	61.3	61.2	61.0	60.9	60.4	60.2
Ethyl 2-methylbutyrate	62.4	62.3	—	62.1	62.0	61.4	61.3
2-Methylbutyl acetate	68.6	68.1	68.0	67.9	67.8	67.2	67.1
1-Hexanol	69.0	68.4	68.4	68.3	68.1	67.6	67.4
trans-2-Hexen-1-ol	—	68.9	68.8	68.7	68.6	68.1	67.9
1-Hexyl acetate	—	103.6	104.1	103.9	103.1	102.4	102.3

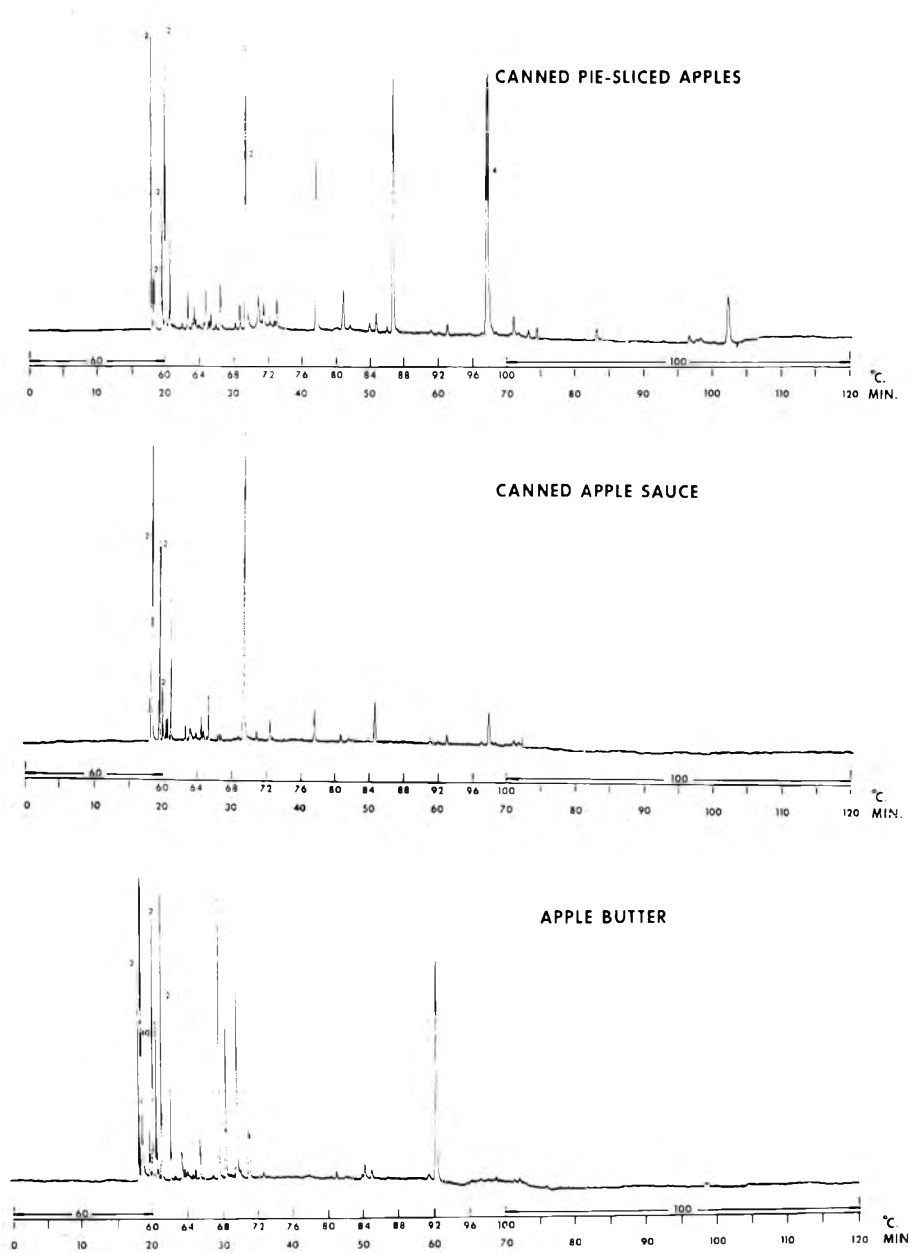

Fig. 5—Apple products: a) canned pie-sliced apples, b) canned apple sauce, c) apple butter.

comparisons, for juice blending and for evaluation of aroma loss and change during processing operations.

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Degradation of DDT and DDE by Cheese Microorganisms

SUMMARY—Representative microorganisms of the diverse flora associated with several varieties of surface-ripened cheese were isolated. Species of streptococci, micrococci, yeasts and Gram-positive rods accounted for most of the isolates. These were incubated at 32°C in media containing 0.5 ppm lindane, p-p DDE, and p-p DDT. The pesticides were dissolved in a small amount of ethanol to facilitate incorporation into the media. Following incubation for 10 days, the concentrations of residual pesticides were determined by electron capture gas chromatography. The growth of streptococci and micrococci isolates did not apear to produce any changes in pesticide levels, though they apparently were uninhibited by the pesticides. However, the growth of other isolates dechlorinated p-p DDT. Moreover, aerobic growth of geotrichum species caused almost complete disappearance of the p-p DDT and p-p DDE peaks from the gas chromatograms. These results suggest the possibility that residues of DDT and DDE might be degraded if they are present in certain types of surface-ripened cheeses.

INTRODUCTION

HISTORICALLY, chlorinated hydrocarbon pesticides have been considered very resistant to physical and microbial degradation. Their unaltered presence in soils for many years has been noted (Alexander, 1965). Reports of microbial degradation of chlorinated hydrocarbon pesticides are few, but they have been appearing in increasing numbers recently (Chacko et al., 1966, 1967; Guenzi et al., 1967; Matsumura et al., 1967). For the most part, only soil microbes have been observed to be active in degradation (Matsumura et al., 1967). Microorganisms associated with food fermentations have not been reported to degrade pesticides.

Feeds containing low levels of insecticides are sources of residues in the milks of lactating cows (Crosley et al., 1967; Laben et al., 1966). Recently, a Food and Drug Administration official, commenting on the new tolerance for DDT and its analogs in dairy products, mentioned that this insecticide is so widely distributed in the environment that it is impractical to prevent exposure of dairy animals to it. (Duggan et al., 1967).

Inasmuch as a tolerance presently exists for DDT and its analogs in milk, and since cheese technology involves a concentration of fat-soluble materials, it is entirely conceivable that residues of this pesticide might be present in cheeses.

Our laboratory has been engaged in studying the effects of food processing methods on pesticide residues in foods. Previously, we had isolated a large number of microorganisms associated with the diverse flora of soft types of cheese. These facts prompted us to study the effect of the growth of representative types of microorganisms isolated from the surfaces of commercial samples of Roquefort, Blue, Limburger, Liederkranz and Brick cheeses on concentrations of lindane, DDE and DDT in growth media.

EXPERIMENTAL

Cheese samples

Packages of Roquefort, Blue, Limburger, Liederkranz and Brick cheeses were purchased at retail stores in Ithaca, N.Y.

Media

Plate counts at 32°C of surface and interior portions of cheeses were made on several media. The APT medium of Evans et al. (1951) was used to estimate the total bacterial population. A TYE medium of the following composition was used: Tryptone (Difco), 10 g; yeast extract (Difco), 5 g; K_2HPO_4, 5 g; distilled water 1 l; pH 7.0 to 7.2. A salt medium (TYES) was prepared by supplementing TYE with 9.5% NaCl. An azide-dextrose medium (Difco) was used for the selective estimation of streptococci.

Representative isolates were incubated at 32°C in TYES broth containing 0.5 ppm lindane (gamma isomer of 1,2,3,4,5,6-hexachlorocyclohexane), p-p DDT [1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane], and p-p DDE [1,1-dichloro-2,2-bis(p-chloro-

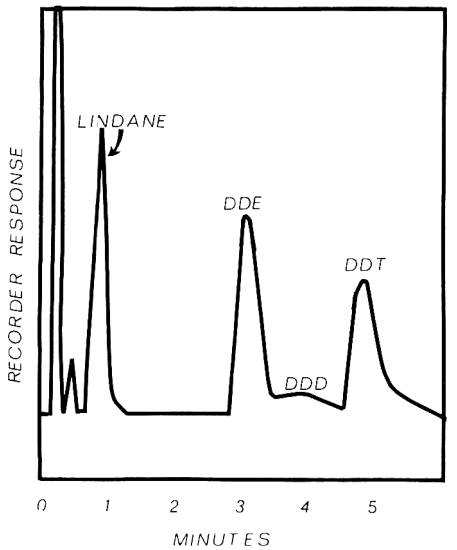

Fig. 1—Gas chromatogram of an uninoculated medium containing 0.5 ppm lindane, DDE, and DDT. The medium was incubated at 32°C for 10 days.

phenyl)ethylene]. The pesticides were dissolved in a small amount of ethanol to facilitate incorporation into the media. Following aerobic incubation for 10 days, the concentrations of residual pesticides were determined by electron capture gas chromatography.

Pesticide analysis

Extraction and clean-up procedures described by Onley (1964) were used to prepare samples for gas chromatographic analysis. Five μl of purified samples were injected into the gas chromatograph (Aerograph Model 204-1). Peak areas were estimated by triangulation.

RESULTS & DISCUSSION

MICROCOCCI, STREPTOCOCCI, and Gram-positive rods comprised most of the microorganisms associated with the flora of surface-ripened cheeses (Table 1). In lower, but significant numbers, were yeasts. The growth of geotrichum species is very important in the ripening of surface-ripened cheeses. These organisms metabolize the lactic acid at the cheese surfaces, thus raising the pH sufficiently so that salt-tolerant bacteria can grow.

Table 1—Microscopic examination of types of microorganisms isolated from soft types of cheese.

Isolate[1]	Approximate %
Micrococci	22
Streptococci	36
Yeasts	6
Gram-positive rods	36

[1] Gram stains were made of representative colonies.

Selective plating media were used to further characterize the flora (Table 2). The incidence of pesticide-degrading isolates was highest in the case of Liederkranz cheese. The surface contained greater viable numbers, as expected, and most of the surface flora was salt-tolerant. Streptococci were present in substantial numbers on the surfaces of Liederkranz cheese.

Representative colonies were isolated and incubated in the pesticide-containing medium. Large, distinct peaks were evident on electron capture gas chromatographic analysis of the purified, uninoculated control sample (Fig. 1). The growth of many isolates had no effect on the peak areas of the three pesticides. However, the

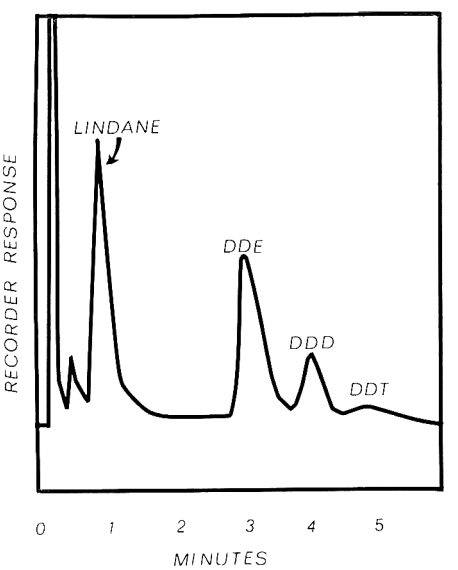

Fig. 2—Gas chromatogram of the growth medium of Brevibacterium linens *isolate 16AE2. The medium initially contained 0.5 ppm lindane, DDE and DDT. Incubation was at 32°C for 10 days.*

metabolism of isolate 16AE2, a Gram-positive, short rod, resulted in a decrease of the DDT concentration and an increase in the level of DDD (Fig. 2). This dechlorination has been observed with some soil and sewage microorganisms (Guenzi et al., 1967); however, it has not been reported previously for food microorganisms. The peak area for lindane was unchanged indicating no activity on this pesticide. Indeed, the nearly constant peak areas for lindane on the gas chromatograms of all samples provided excellent internal control data. The growth effect of geotrichum on the concentrations of DDE and DDT was more striking (Fig. 3). Significant reductions in both the p-p DDT and p-p DDE peaks were apparent indicating degradation of these pesticides to unknown compounds. Our laboratory

Table 2—Plate counts of commercial Liederkranz cheese on different media.

Medium	Cheese area sampled, colonies/g	
	Interior	Surface
APT	410×10^5	166×10^7
AzD	3×10^3	48×10^7
TYE	128×10^5	172×10^7
TYES	12×10^3	166×10^7

[1] See text for composition of media.

Table 3—Summary of characteristics of bacterial isolates 16AE1 and 16AE2.[1]

Characteristic	16AE1	16AE2
NH_4 as sole nitrogen source	–	–
NH_3 produced in broth	+	+
Indole	–	–
Casein hydrolysis	+	+
Starch hydrolysis	+	+
Litmus milk (11 days)	Reduced	Reduced
Acid production		
Glucose	–	–
Lactose	–	–
Maltose	–	–
Sucrose	–	–

[1] On agar, both form slime and produce soluble dark yellow pigments. Both are aerobic, nonspore formers. Optimum growth temperature range is 25–30°C.

is presently endeavoring to establish the identity of the breakdown products.

Physiological characteristics of the active isolates were examined (Table 3). Isolates 16AE1 and 16AE2 have characteristics similar to *Brevibacterium linens* and 16AA4 possesses distinct characteristics of species of geotrichum (Table 4).

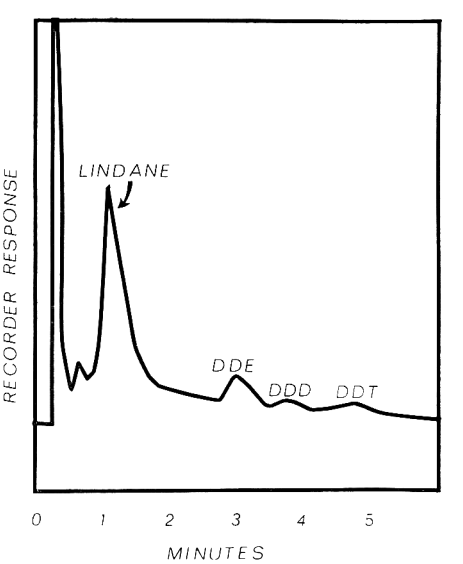

Fig. 3—Gas chromatogram of the growth medium of geotrichum isolate 16AA4. The medium initially contained 0.5 ppm lindane, DDE and DDT. Incubation was at 32°C for 10 days.

Table 4—Summary of characteristics of geotrichum isolate 16AA4.[1]

Characteristic	
NH_4 as sole nitrogen source	+
Produce NH_3	+
Casein hydrolysis	−
Starch hydrolysis	−
Acid production	
Glucose	−
Lactose	−
Maltose	−
Sucrose	−

[1] White cottony growth is produced on agar. Microscopically, large, thick-walled septate filaments are observed. Spores are not formed.

Table 5—Residual amounts of DDE, DDD, and DDT following incubation of cheese microorganisms with DDT and DDE.

Microorganism	DDE[1]	DDD[1]	DDT[1]
Control	11.8	0.3	11.2
Geotrichum 16AA4	2.3	0.3	1.8
Geotrichum candidum ATCC 12784	4.0	1.6	2.2
Brevibacterium linens ATCC 9172	6.0	1.9	2.0
Brevibacterium linens ATCC 9175	5.2	3.7	1.7

[1] Measured peak areas, cm^2.

Table 6—Relative amounts of DDE, DDD, and DDT following incubation of Brevibacterium linens isolated from soft-types of cheese with DDT and DDE.

Isolate	DDE[1]	DDD[1]	DDT[1]
Control	9.1	0.3	8.3
16AE1	6.5	1.9	3.1
16AE2	7.6	2.9	0.5

[1] Measured peak areas, cm^2.

This organism will be classified further.

The initial findings were confirmed by results of additional experiments (Table 5). Growth of our geotrichum isolate, 16AA4, was most effective in reducing levels of DDE and DDT, and DDD was not formed. Growth of *Geotrichum candidum* ATCC 12784 was effective also in reducing the concentrations of these pesticides, confirming results of research with our isolate, 16AA4. Likewise, the growth of cultures of *B. linens*, obtained from the American Type Culture Collection, reduced the concentrations of DDE and DDT. Formation of more DDD by these cultures was observed. Similar results were obtained with isolates of *E. linens* (Table 6). Evidently, aerobic growth of *B. linens* degrades DDE but to a lesser extent than does the growth of geotrichum species.

Table 7—Effect of pH on the degradation of DDE and DDT by geotrichum isolates.

pH	Microorganism	DDE[1]	DDD[1]	DDT[1]
5.5	Control	10.5	0.3	6.8
	Geotrichum 16AA4	8.7	1.5	3.8
	Geotrichum candidum ATCC 12784	9.5	6.0	0.3
7.0	Control	10.0	0.6	6.4
	Geotrichum 16AA4	7.2	2.7	2.0
	Geotrichum candidum ATCC 12784	9.0	6.0	0.2
8.5	Control	10.3	0.7	5.9
	Geotrichum 16AA4	7.0	3.5	1.3
	Geotrichum candidum ATCC 12784	9.0	6.2	0.2

[1] Measured peak areas, cm^2.

Degradation of DDE and DDT by the growth of geotrichum was observed over a wide range of pH (Table 7). This organism grew well from pH 5.5 to 8.5. More degradation was observed at pH 8.5 than at pH 5.5.

Accumulation of DDT and dieldrin by microorganisms from culture fluids has been reported (Chacko et al., 1967). This observation prompted us to determine if cells of our geotrichum isolate bind DDT, DDE, and DDD possibly resulting in low recoveries in the extraction process. Data of several experiments indicate that binding is not involved.

Results of these experiments suggest the possibility that residues of DDT and DDE might be degraded if they are present in certain types of surface-ripened cheeses. Being aerobic, the brevibacterium and geotrichum are unable to grow in the interior areas of the cheese. However, perhaps the pesticide-degradation activity, if formed on cheese surfaces, is capable of diffusing into the central areas of cheese in much the same manner as the cheese-ripening proteolytic enzymes produced by the surface flora.

A continued study of the pesticide-degrading food microorganisms is contemplated. More detailed information is desirable regarding their taxonomy, their physiological characteristics (especially their proteolytic potential), and the nutritional factors as well as the kinetic aspects of the production of the pesticide-degrading activity.

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