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CONTENTS

Effect of Radiation Environment on the Thermal Resistance of Irradiated Spores of Clostridium Sporogenes P.A. 3679

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SUMMARY

The thermal resistance at 100°C of *Clostridium sporogenes* P.A. 3679 was determined after the spores had been irradiated with gamma rays or cathode rays under various environmental conditions.

The pre-irradiation treatment sensitized the spores to heat, and spores irradiated either in air or under vacuum were heat-sensitized to the same extent. The heat sensitization induced was slightly greater when the spores were irradiated at a pH near neutrality, than at an acid pH. Spores suspended in phosphate buffer (pH 7.0) were heat-sensitized to a greater degree than spores suspended in nutrient broth or pureed ham. The thermal resistance of the irradiated spores was not influenced by the concentration of viable spores in the suspension (phosphate buffer), but as the proportion of dead cells and dead spores increased with increasing doses of irradiation, the thermal resistance also increased.

Spores irradiated with 660,000 rep at 66–68°C were more heat-sensitive than spores irradiated at room temperature or in the frozen state. Below this irradiation level this effect was not evident.

INTRODUCTION

Early studies by Curran and Evans (1938) and more recent investigations by Morgan and Reed (1954), Kempe (1955), and Kan *et al.* (1957) have shown that irradiating certain bacterial spores sensitizes them to the effect of the lethal action of heat. Kempe *et al.* (1959) applied this combination of processing treatments to sterilize cans of ground beef inoculated with spores of *Clostridium sporogenes* P.A. 3679. They reported that the F_0 required to produce commercially sterile cans after irradiation by 1.3–1.8 megarep was approx ¼ that required when only heat-processing was used.

Kempe *et al.* (1960) also used combined irradiation–heat processing to preserve canned green peas inoculated with spores of *Clostridium sporogenes* P.A. 3679. The F_0 required to sterilize the peas was 0.5 following 1.2 megarad of gamma radiation, and about 4–5 with unirradiated peas.

The adverse changes in food brought about by irradiation at high dose levels can, in some instances, be decreased by making certain environmental changes (atmosphere, temperature, etc.) in the food during irradiation. It is not known, however, whether the heat-sensitization effect of irradiation is influenced by these environmental changes during the irradiation treatment.

It would also be of interest to learn whether other factors such as the nature and pH of the substrate, and the concentration of spores surviving irradiation altered the heat-sensitization effect of irradiation.

Therefore, the main objective of this investigation was to determine whether altering the environment would alter irradiation changes in the heat sensitivity of spores of *Clostridium sporogenes* P.A. 3679 and *Bacillus subtilis* A.T.C.C. 6633. These two

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organisms were selected because of their importance in causing spoilage of canned whole hams. Since slow heat transfer through the ham makes it impractical to sterilize a canned whole ham by the conventional method of steam processing, it is felt that this product might be greatly improved by this new technique of sterilization, if this process proves applicable.

This paper presents only the results with *Clostridium sporogenes* P.A. 3679. Results with *Bacillus subtilis* will be reported later.

EXPERIMENTAL METHODS

Propagation of P.A. 3679 spores. Lyophilized spores of *Clostridium sporogenes* P.A. 3679 (ATCC No. 7955) were obtained from the American Type Culture Collection, Washington, D. C. An actively growing culture, obtained by successively inoculating increasingly larger flasks of media, was transferred to a 4-L Erlenmeyer flask containing a pork infusion medium together with cooked, ground, lean pork and several iron nails. After incubation for 1 wk at 37°C and 2 wk at 30°C, the spores were harvested by centrifugation, washed several times with distilled water, resuspended in phosphate buffer, pH 7.0, and stored at 2–4°C until used.

The pork infusion medium was prepared by simmering ground lean pork (1 lb/L) for 1 hr in distilled water. The broth was filtered through cheesecloth, any fat was skimmed off the surface, and water lost during evaporation was replaced. The following ingredients were added to each liter of infusion: 5 g Difco peptone, 1.5 g Difco tryptone, 1 g dextrose, and 1.25 g K_2HPO_4. After the ingredients were dissolved, the pH of the infusion was adjusted to 7.2–7.4 with KOH.

Three individual spore crops were cultivated throughout the course of this investigation. One spore crop was propagated on a medium quite similar to the pork infusion medium except that beef liver was substituted for lean pork. These spores had in phosphate buffer at 100°C a lower D value (about 130 min) than spores grown in pork infusion medium (about 185 min), and therefore were discarded.

Sample preparation. For the studies related to the effect of atmosphere of irradiation on heat sensitization, the spores were suspended in phosphate buffer (pH 7.0), poured into 5-ml Pyrex ampoules, heat-shocked 15 min at 100°C, and cooled.

Suspensions to be irradiated in a normal atmosphere were sealed in ampoules with a 3-way burner flame. Suspensions to be irradiated under a low oxygen tension were sealed in ampoules after the headspace was evacuated with a mechanical vacuum pump to a pressure of about 1 mm Hg. For spores to be treated in an atmosphere of nitrogen gas, the headspace gas was removed by mechanical vacuum pump and replaced with pyrogallol-washed nitrogen gas. This cycle was repeated four times before the ampoule was sealed.

In studies related to the effect of pH of the substrate, three levels of pH (4.5, 5.8, and 7.0) were investigated. The buffer substrates were prepared by blending solutions of potassium acid phosphate and disodium phosphate in the proper proportions for making Sorensen's buffer mixtures.

Three different substrates were investigated: phosphate buffer (pH 7.0), nutrient broth, and a ham puree. The nutrient broth was prepared from dehydrated Difco nutrient broth. The ham puree was prepared by grinding lean ham in a sterile meat grinder, blending for several minutes in an Osterizer with an almost equal portion of water, and further comminuting the slurry in a ball mill. Portions of the puree were poured into screw-cap jars and held at −18°C until used.

Three different temperatures during irradiation were investigated: room temperature (20°C), −78°C, and 66–68°C. For irradiation at room temperature, 2.5 ml of the heat-shocked spore suspension (phosphate buffer pH 7.0) were pipetted into a 50-mm-diameter Petri dish. The dish was covered with Saran film and irradiated with cathode rays. For samples irradiated in the frozen state, the Petri plate was surrounded with crushed dry ice. For samples irradiated at 66–68°C, the Petri plate was partially immersed in hot water until desired sample temperature was reached. The irradiated suspension was immediately cooled by placing the Petri plate on ice.

Irradiation sources. Most of the irradiation was carried out with gamma rays from a cobalt-60 source with an output of approx 200,000 rep/hr (186,000 rad/hr). When the effect of temperature during irradiation was studied, cathode rays were used from a General Electric resonant transformer (Knowlton *et al.*, 1953) rated at one million electron volts. This machine could deliver up to one million rep in about 1 sec.

Method for determining thermal resistance. The equipment for heating the spores after irradiation, described by Stern and Proctor (1954), basically consists of a thermostatically controlled (±0.5°C) heating bath of mineral oil and a cooling bath of ice water. A small amount (0.025 ml) of spore suspension is sealed in melting-point capillary tubes (Kimex brand, Owens-Illinois Glass Co., Toledo, Ohio), and the tubes are positioned by a sample holder. At the end of the prescribed heating time the tubes are automatically and rapidly (0.4 sec) transferred from the heating bath to the cooling bath. The spore suspension was introduced

into the capillary tube with a Warburg-manometer calibrator (Micro-Metric Instrument Co., Cleveland, Ohio) fitted with a glass adapter and 24-gauge hypodermic needle. The heating lag (time required to reach 0.1°C below heating temperature) in these capillary tubes, both by the equations of Olson and Schultz (1942) and by measurements with a thermocouple attached to a Speedomax (Leeds and Northrup Co., Philadelphia, Pa.), was 10–12 sec. To compensate for this heating lag, all capillary tubes, including controls, were heated for an additional 10 sec.

Spore counting. After heating, the capillary tubes were washed in petroleum ether, soaked several minutes in chromic acid cleaning solution, and finally rinsed under cold tap water. Each capillary tube was placed in a culture tube containing 10 ml water and was crushed with a glass rod.

Counts were made to determine the number of surviving spores by inoculating (in triplicate) deep-culture Pyrex tubes (12 mm OD, 200 mm long) containing 0.5 ml of a sterile 5% sodium bicarbonate solution with an appropriate amount of the diluted spore suspension. Approx 12 ml of an anaerobic culture medium was then added, followed by stratification with a 2% agar solution. Colonies were counted after 18–24 hr at 37°C.

Composition of the culture medium was: 250 ml beef liver infusion (prepared by simmering 1 lb of ground beef liver for 1 hr with 1 L of water, filtering, and adjusting the pH of the infusion to 7.1–7.3 with KOH); 750 ml distilled water; 15 g agar; 29.3 g dehydrated fluid thioglycollate medium with resazurin indicator.

This medium gave as high counts with P.A. 3679 spores as did Andersen's medium (1951), and it was specifically developed for its ease of preparation.

Analysis of the data. Survival curves of the spores after heating were drawn by averaging arithmetically the three individual anaerobic-tube colony counts for each heated capillary tube and plotting the logarithms of the average counts as a function of heating time. In most instances, the death rate became constant only after a certain heating time had elapsed. Therefore, regression analysis was performed only on data from that part of the survival curve in which survival was linear with respect to time. Five capillary tubes were heated at each heating time, and the regression analysis was carried out on the survival data of three successive heating times. The slope of the regression line represents the thermal death rate (or death rate constant), and the negative reciprocal of the slope is the decimal reduction time or D value.

To determine whether there was a significant difference in the thermal death rates of P.A. 3679 spores irradiated under two different conditions, the various replicate values of regression slopes obtained for a given irradiation-heating condition were pooled by applying Hald's (1957) weighted formula to determine the average regression slope. Two average regression slopes thus obtained could be statistically compared by a "t" test.

RESULTS AND DISCUSSION

Fig. 1 shows the effect of gamma irradiation on the decimal reduction time (D value) at 100°C of spores of P.A. 3679 (Crop 1). Before being heated, these spores had been irradiated in various atmospheres in a phosphate buffer substrate (pH 7.0) at room temperature. For a given irradiation dose, the D values of the spores irradiated in air or under vacuum (1 mm Hg) were not significantly different. When the spores were irradiated with 660,000 rep in a nitrogen atmosphere the thermal resistance increased significantly. The mean D values for the nitrogen irradiated spores and vacuum irradiated spores were compared by a "t" test and the resulting value of t was significant at the 95% level. A possible explanation why spores irradiated in nitrogen had a

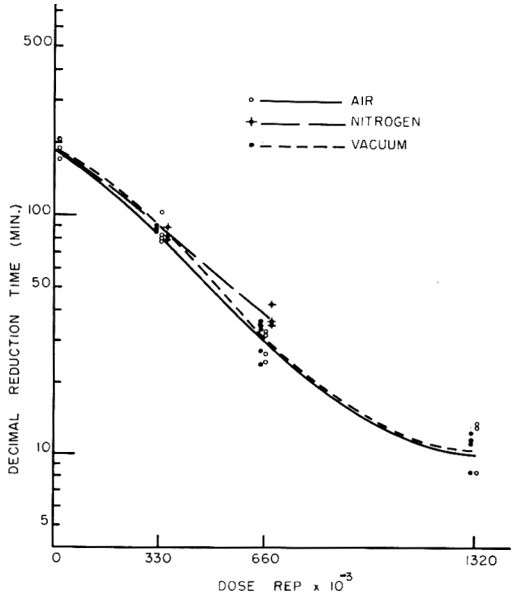

Fig. 1. Effect of atmosphere of gamma irradiation on the decimal reduction of time of P.A. 3679 spores irradiated at room temperature in phosphate buffer pH 7.0 and then heated at 100°C.

greater thermal resistance than spores irradiated in air or at low oxygen tension might be as follows. The technique used in sealing under nitrogen probably removed more oxygen than sealing under vacuum (1 mm Hg). In the radiolysis of water the formation of hydrogen peroxide is enhanced by the presence of oxygen. Thus, there probably was a greater accumulation of hydrogen peroxide in the spore suspensions irradiated in air or at low oxygen tension. It is conceivable that this hydrogen peroxide, coupled with the heat treatment, could cause a greater destruction of the spores.

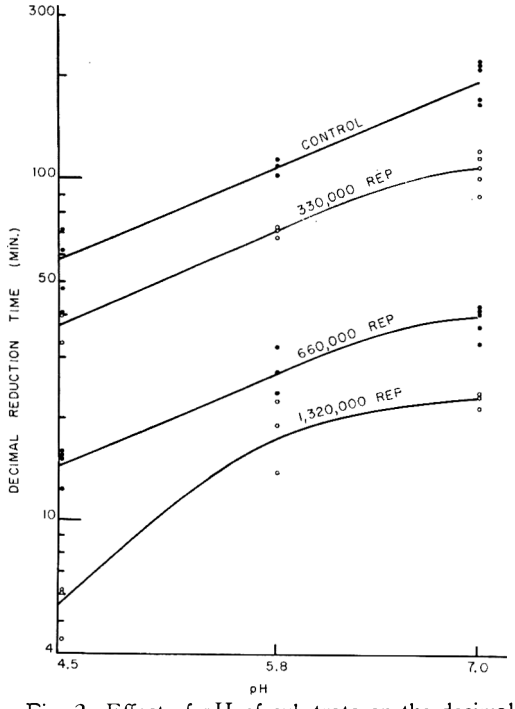

Fig. 2. Effect of pH of substrate on the decimal reduction time of P.A. 3679 spores irradiated in air at room temperature in phosphate buffer and then heated at 100°C.

In Fig. 2 log decimal reduction time has been plotted as a function of pH for various irradiation levels. Before being heated, the spores were irradiated at room temperature in phosphate buffer at various pH levels and in air atmosphere. For the unirradiated spores, decimal reduction time was a linear function of pH. With the irradiated spores, however, the rate of increase in D value dropped off as pH approached neutrality. This may indicate that combined irradiation-heating is more effective at a pH near neutrality than in the acid range.

An attempt was made to use approx the same concentration of viable spores throughout this investigation. The concentration of viable spores following irradiation was usually 200,000–800,000/ml. Since each capillary tube contained 0.025 ml of spore suspension, the initial count for the heating runs was 5,000–20,000 spores per tube.

Fig. 3 shows the effect of spore concentration on the D value at 100°C of irradiated and unirradiated spores (Crop 2). The upper curve was determined with the usual spore concentration, 200,000–800,000/ml, and the lower curve with 1/10 that concentration range. Spore concentration did not significantly affect thermal resistance of unirradiated or irradiated spores until irradiation treatment was 660,000 rep or greater. Then thermal resistance was significantly lower (95% level) with lower spore concentrations. In order to have approximately the same concentration of spores surviving the irradiation treatment, a more

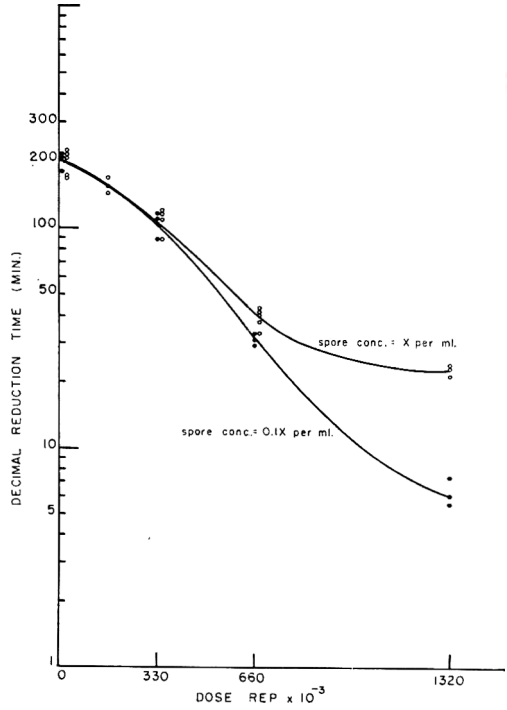

Fig. 3. Effect of gamma irradiation on the decimal reduction time of P.A. 3679 spores at two different concentrations, irradiated in air in phosphate buffer pH 7.0 at room temperature and then heated at 100°C.

concentrated spore suspension had to be used with increasing irradiation dose. Thus, a greater number of dead cells and spores were present in the suspensions (phosphate buffer) after treatment with the higher irradiation doses and these dead cells, which may have ruptured and released intracellular material, probably exerted a protective influence on the remaining viable spores during heating.

Fig. 4 plots decimal reduction time of P.A. 3679 (Crop 3) as a function of heating temperature. These spores had been suspended in phosphate buffer (pH 7.0) and irradiated in air at room temperature. A line through the D values is known as a phantom thermal death-time curve. The negative reciprocal of the slope of the thermal death-time curve represents the Z value, and this parameter is an indication of the thermal resistance of an organism in a particular substrate over the temperature range at which it was determined.

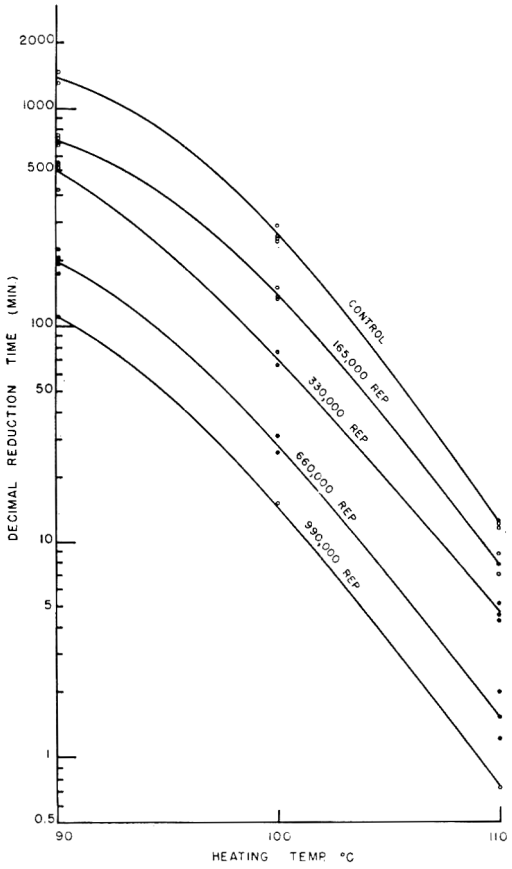

Fig. 4. Phantom thermal death-time curves of gamma-irradiated and unirradiated spores of P.A. 3679 suspended in phosphate buffer pH 7.0.

In order to compare the phantom thermal death-time curves, each was drawn as the best curve passing through the average D value for each given temperature. This does not necessarily imply that the thermal death-time curve for P.A. 3679 is nonlinear over the temperature range of 90–110°C. Also, for comparative purposes, Z values were measured over the interval of 100–110°C. These Z values were:

Irradiation treatment prior to heating	Z value
0 rep	13.5°F
330,000 rep	14.4°F
660,000 rep	15.2°F
990,000 rep	13.6°F

These Z values do not appear to be significantly different; thus it can be stated that irradiation prior to heating changed the F value but did not affect the Z value.

With regard to the actual shape of the thermal death-time curve of P.A. 3679 in the low-temperature range, there is a paucity of information in the literature to elucidate this. However, Halversen and Hays (1936) reported thermal death times for *Cl. botulinum* spores in various foods. Thermal death-time curves constructed from their data did show a change in slope, and the slope was much less steep over the range of 90–100°C than over 100–110°C.

Fig. 5 compares the D values obtained when irradiated P.A. 3679 spores (Crop 3) were heated in either phosphate buffer (pH 7.0), nutrient broth (pH 6.7), or ham puree (pH 6.2). The unirradiated spores showed greatest thermal resistance in ham puree, and least in nutrient broth. When the spores were suspended in ham puree or nutrient broth, irradiation heat-sensitized them to about the same degree (the two curves are essentially parallel). However, irradiation sensitized the spores to heat to a greater degree in phosphate buffer than in the other two substrates. Thus, some organic or inorganic component of the ham puree and nutrient broth protected P.A. 3679 spores during irradiation. Kempe (1955) reported

that irradiation of *Cl. botulinum* prior to heating decreased the F_0 value, and this reduction of F_0 value was less in gelatin or nutrient broth than in phosphate buffer (pH 7.0).

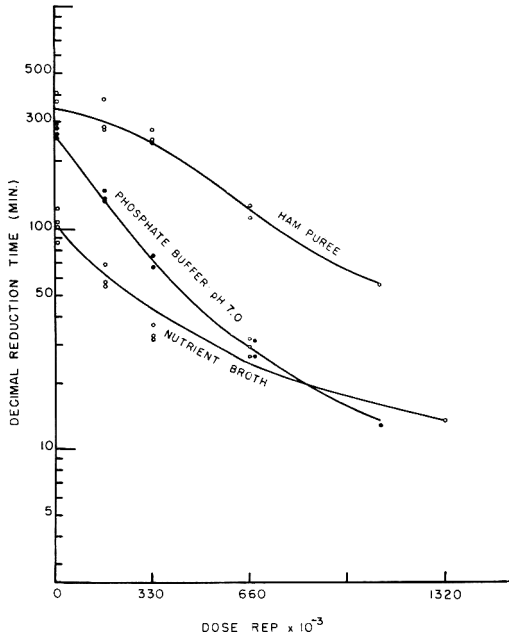

Fig. 5. Effect of gamma irradiation on the decimal reduction time of P.A. 3679 spores suspended in various substrates, irradiated in air at room temperature, and then heated at 100°C.

Fig. 6 shows the effect of temperature during irradiation on the thermal resistance of P.A. 3679 (Crop 3).

In comparing the thermal resistances of spores irradiated in phosphate buffer at different temperatures, there was no apparent difference between −78°C and 20°C over a dose range of 0 to 660,000 rep. Further, there was no significant difference between 20°C and 66–68°C with either 165,000 or 330,000 rep. With 660,000 rep, however, 66–68°C gave a marked decrease in heat resistance below that from 20 or −78°C.

In order to offer an explanation as to why the spores irradiated (660,000 rep) at 66–68°C were more heat-sensitive than the spores irradiated at 20°C, one must first consider the probable mechanism of the radiation-induced sensitivity. It has been suggested (Giese and Crossman, 1946; Adams and Pollard, 1952) that irradiation damages or breaks certain bonds within some functional protein, and when the cell is subjected to heat the thermal energy causes excitation and vibration, with either subsequent rupture of the protein molecules or sufficient unfolding of the main chains that the necessary biological configuration is destroyed. It is conceivable, therefore, that if the radiation was applied while the molecule was in a high state of thermal excitation, the amount of chemical-bond damage would be greater than when the molecule was irradiated in a low state of thermal excitation. Thus, the thermal requirements for denaturing the protein following irradiation would be lowered.

Freezing the spores to −78°C did not affect their heat resistance. The D value at 100°C for unirradiated spores frozen and thawed before heating was not significantly different from the D value for unfrozen spores.

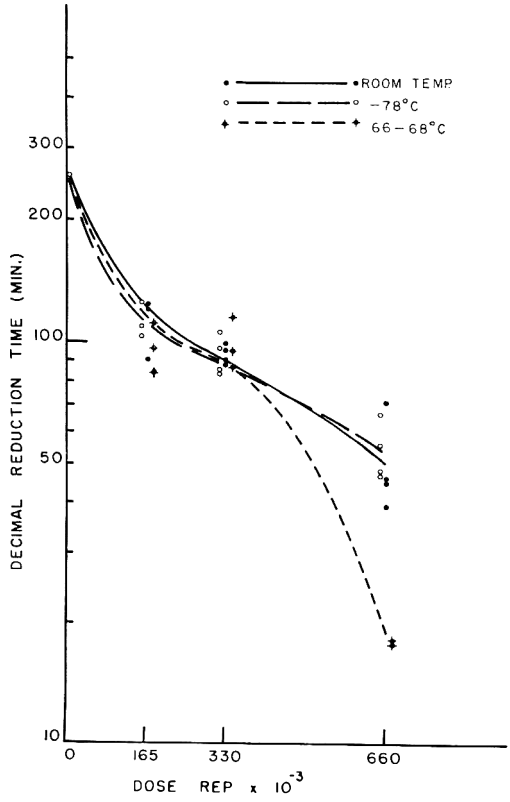

Fig. 6. Effect of cathode-ray irradiation on the decimal reduction time of P.A. 3679 spores suspended in phosphate buffer pH 7.0, irradiated in air at various temperatures, and then heated at 100°C.

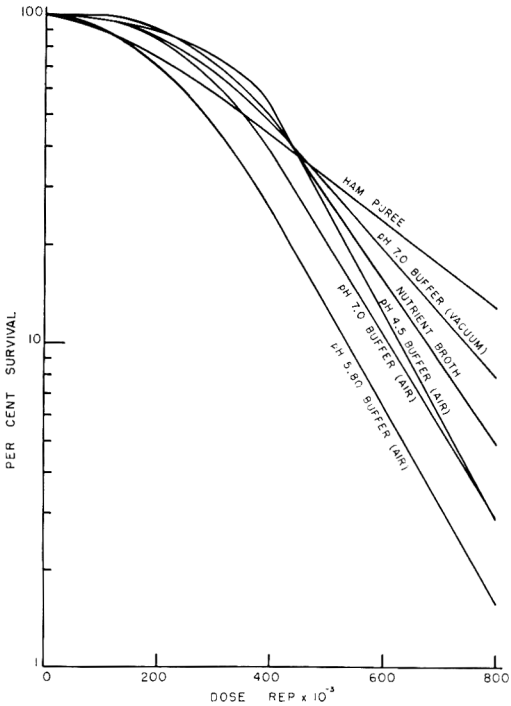

Fig. 7. Survival curves for P.A. 3679 spores irradiated at room temperature in various substrates and atmospheres with gamma rays.

Fig. 7 represents the survival curves for P.A. 3679 spores (Crop 3) irradiated with gamma rays under a variety of conditions. Destruction of the spores by irradiation was not a straight-line function from the start of irradiation, but became linear after the spores had received a dose of several hundred thousand rep.

The irradiation dose required to destroy 90% of the spores is referred to herein as the D_{90} dose, or decimal reduction dose. This value was arrived at for the various survival curves by performing a regression analysis on the survival points that appeared to fall on a straight line. The negative reciprocal of the slope of the survival curve expressed in rep is the D_{90} dose. The regression analysis was carried out over the dose range of 400,000–800,000 rep. The D_{90} doses thus obtained were:

		D_{90} dose
Phosphate buffer pH 7.0	air	350,000 rep
Phosphate buffer pH 5.8	air	330,000 rep
Phosphate buffer pH 4.5	air	312,000 rep
Phosphate buffer pH 7.0	vacuum	510,000 rep
Nutrient broth	air	397,000 rep
Pureed ham	air	758,000 rep

The D_{90} dose apparently decreased as the pH decreased; however, when the slope of the pH 7.0 survival curve was compared by t-test with the slope of the pH 4.5 survival curve, they were not significantly different at the 95% level.

A significant difference at the 95% level was found to exist between D_{90} doses for irradiation in air and irradiation under vacuum.

The extreme resistance of P.A. 3679 in pureed ham may be due to the competition by some component of the ham puree for the free radicals formed during irradiation.

Fig. 8 gives the survival curves of P.A. 3679 suspended in phosphate buffer (pH 7.0) and irradiated at various temperatures with cathode rays.

The D_{90} doses at the various temperatures of irradiation were: 171,000 rep at 20°C, 196,000 at 66–68°C, and 260,000 at −78°C.

A comparison of the slopes of the survival curves resulting from irradiation at 20°C and −78°C revealed a significant difference at the 95% level.

The difference in D_{90} doses for spores irradiated at 66–68°C and at 20°C was not significant at the 95% level.

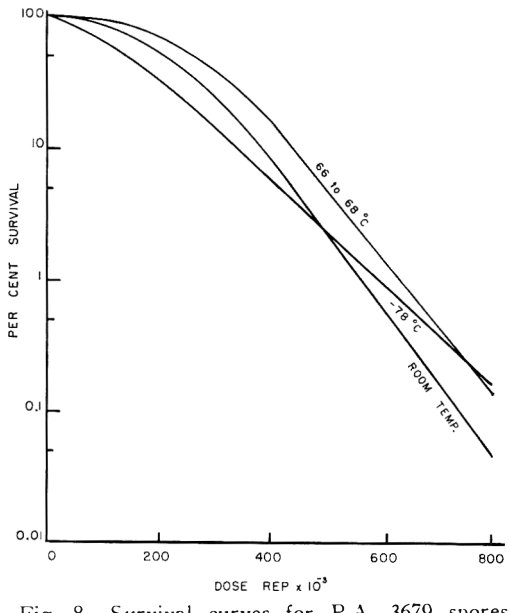

Fig. 8. Survival curves for P.A. 3679 spores irradiated at various temperatures in phosphate buffer pH 7.0 with cathode rays.

ACKNOWLEDGMENT

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Chemical Events During Death of Bacterial Endospores by Moist Heat[a]

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SUMMARY

Efforts were continued in an attempt to assess quantitatively the behavior of certain cellular components while the spores were being exposed to moist heat, and to correlate such behavior with the thermal death reaction.

Spores of *Bacillus subtilis* were preheated over prolonged intervals in an attempt to induce the release of cellular dipicolinic acid (DPA) without exercising serious loss of viability, and then to evaluate the subsequent effect of DPA release upon the thermal death rate of the preheated spores. At 45°C in 2.5m*M* PO_4 buffer at pH 7, the net DPA released amounted only to 1.6 and 2.7% at the end of 20 and 30 hr. The treated spores exhibited no loss in viability and when heated at 98.5°C in 25m*M* PO_4 buffer at pH 7, exhibited no difference in their thermal death rates. Prolonging the heat treatment up to 9 days did not materially change the final results. To induce faster and greater DPA release, the above experiment was repeated at 80 and 90°C. At 80°C the net DPA released was 3.6 and 4.1% at the end of 3 and 8.5 hr, without loss in viability. However, the treated spores exhibited some reduction in their thermoresistance at 98.5°C. At 90°C greater amounts of DPA were released, but were associated with substantial loss in viability, and the surviving spores exhibited marked reduction in their thermoresistance at 98.5°C. These observations suggest the existence of DPA in more than one structural form; free or loosely bound to the spore structure and easily exuded upon mild heating or even standing under refrigeration; and a second more strongly bound form which required severe heating for its release. It is this latter form which might be associated with the mechanism of thermoresistance. Along with DPA, following the same pattern but at much higher rates, ninhydrin-positive material was exuded during sublethal heating at 80, 85, and 90°C. It was suggested that such material might be of similar nature to that exuded during germination of *Bacillus* species as described by Powell (1957).

Spore suspensions of *Bacillus coagulans* (thermoacidurans) of varied thermostability were heated in 25m*M* phosphate buffer at pH 7, at 95°C, and both the rate of death and DPA release were established. Results showed a difference in kinetics between the two reactions. Death progressed at higher rates than DPA release. Higher rates of death were associated with higher rates of DPA release. Spores of same strain were heated in both water and 10m*M* glycylglycine, a death accelerating agent, at 100°C, sampled at intervals and analyzed for survival, dry weight, calcium, manganese, magnesium and DPA. Results showed the death of spores and their concurrent exudation of DPA and divalent cations were both markedly accelerated in the presence of glycylglycine. The 25-min survival and exudate levels in glycylglycine were near equivalent to those at the 65-min levels in water.

The kinetics of death and the concurrent release of DPA and calcium were further assessed in more detail in 5m*M* phosphate buffer at pH 7 at 96 and 99°C using spores of *B. coagulans* (thermoacidurans). Death again progressed at a higher rate than that of either DPA or calcium release. Based on the molar ratio of Ca/DPA released in the supernatant, the initial ratio was always greater than 1, then rapidly dropped and eventually plateaued at a value of

less than 1. Similar patterns of death and calcium and DPA release were obtained for *B. cereus* when heated at 86°C.

All of the above observations demonstrate the association of the thermal death reaction with the exudation of ninhydrin-positive material, DPA and divalent cations into the heating menstruum. The exact relationship between such cellular components and the spore mechanism of thermoresistance remains inconclusive.

The phenomenon of thermoresistance of bacterial endospores is of the utmost significance to the food technologist. Canning technology bases its preservative action on thermal destruction of the major intoxication and spoilage bacteriospores commonly associated with the food and/or the food container. Understanding the mode of thermoresistance of such spores would effectively contribute toward perfecting a thermal process with maximum lethal effect on the spores and minimum damage to product quality.

A widely accepted concept of bacterial death by heat is thermal denaturation of a critical monomolecular proteinaceous site, within the genetic structure of the cell, that affects and regulates its reproduction (Rahn, 1943). Factors that enhance the thermostability of protein would be expected to do likewise to the bacterial cell. This hypothesis provided guidance to most of the approaches used thus far in exploring the unique mechanism of thermoresistance of the spore cell.

Nanninga (1957) showed that proteins such as casein and pepsin had a high affinity for calcium and magnesium. Bier and Nord (1951) and Gorini (1951) demonstrated that calcium salts induced the thermostability of trypsin, serum albumin, and certain bacterial proteases. Curran *et al.* (1943) found that several bacterial spores contained higher levels of divalent cations, particularly calcium, than did their homologous vegetative forms. They also reported that higher concentrations of calcium were associated with higher thermoresistances. Vas and Proszt (1957) found that intact spores of *Bacillus cereus* contained 4.70% of their dry-weight calcium whereas the germinated spores contained only 0.86%. The higher calcium level in the intact spores was associated with higher thermoresistance.

El-Bisi and Ordal (1956a) obtained higher thermal death rates of spores of *B. coagulans* (thermoacidurans) when produced in the presence of higher levels of phosphate. It was postulated that higher levels of phosphate interfered with the availability of the divalent cations to the sporulating cells. On the other hand, the enrichment of calcium and manganese in the sporulating medium produced spores of higher calcium and manganese content, and consequently of higher thermoresistances (Amaha and Ordal, 1957).

Slepecky and Foster (1959) also showed that the content of individual metals in spores was flexible within a wide range and was dependent on the relative concentration of the particular metal in the growth medium. They inferred that the various cations accumulated by the spores were interchangeable. Spores with maximal or minimal metal content were indifferent in their morphology, staining, refractility, and resistance to killing by desiccation, phenol, and UV radiation. However, higher thermoresistances were associated with higher levels of calcium. Spores with maximal manganese or zinc content possessed minimum calcium, and therefore were thermosensitive.

Amaha and Ordal (1957) demonstrated that the presence of chelating agents with high affinities towards calcium and manganese, such as ethylenediaminetetraacetic acid (EDTA), trishydroxymethylaminomethane (TRIS) and glycylglycine (GG) in the heating menstruum accelerated the

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death reaction and thus caused a considerable reduction in the apparent thermal resistance of the spores.

El-Bisi and Ordal (1958) further demonstrated that higher levels of phosphate or GG in the heating menstruum increased the rate of thermal destruction and that the original destruction rates were partially restored by the addition of calcium and manganous salts. Magnesium and monovalent cations were ineffective in this respect.

Powell (1953) demonstrated a unique organic component, 2,6-dicarboxypyridine (dipicolinic acid, DPA), in the spore cell. Though all spores examined since then contained 5–15% of their dry weight as DPA, the homologous vegetative forms had none. The appearance of mature spores in the sporulating culture coincided with the synthesis of DPA, the incorporation of higher calcium level, and the attainment of higher thermoresistance (Perry and Foster, 1955; Collier and Murty, 1957; Hashimoto *et al.*, 1960; Church and Halvorson, 1959). DPA was released in the surrounding menstruum when the spores were germinated or autoclaved (Powell, 1957; Stedman, 1956).

Rode and Foster (1960), in studies on induced release of DPA from spores of *Bacillus megaterium,* showed that practically all the DPA was released during the first few minutes (6 min at 100°C). They were of the opinion that death of spores preceded the release of DPA.

El-Bisi and Ordal (1956) produced spores of enhanced thermal resistance at higher incubation temperatures. Lechowich and Ordal (1960) demonstrated that such spores possessed higher levels of calcium, manganese, and DPA, and/or higher molar ratios of cations to DPA.

All of the above evidence suggests that certain cations as well as DPA are in some way associated with the mechanism of thermoresistance in the spore cell. In the present investigation, efforts were continued to assess quantitatively the behavior of cellular DPA and certain divalent cations during exposure of spores to moist heat, and to correlate such behavior with the thermal death rates.

MATERIALS AND METHODS

Test cultures. Three cultures were used. The first was a laboratory isolate identified as a *Bacillus subtilis* strain. The second was obtained from the American Type Culture Collection as *Bacillus coagulans* (thermoacidurans) strain 8038 (equivalent to the National Canners Association *B. thermoacidurans* strain 43-P), the identity of which was confirmed in our laboratory. The third was a fresh isolate made in our laboratory from garden soil and identified as *Bacillus cereus.* All identification procedures followed those recommended by Smith *et al.* (1952).

All stock cultures were maintained in the spore state at 3°C until used for preparation of the various spore crops.

Preparation of spore suspension. In the case of both *B. subtilis* and *B. coagulans* strains an active inoculum was prepared by transferring it from a stock agar slant into 150 ml broth contained in a 500-ml Erlenmeyer flask, and placing the flask on a rotary shaker (270 rpm) for 16–20 hr. Sterile agar surfaces were prepared in advance in 150-mm Petri plates or 16-oz prescription bottles. To check for contamination, agar plates or bottles were held 24–48 hr at room temperature before inoculation. Agar surfaces were spread with the active inoculum, using 1.5–2 ml per plate or bottle. Cultures were incubated until maximum sporulation was attained.

Growth was then washed off the agar surface with about 10 ml water per plate or bottle; growth usually flaked off readily in about 15–20 min. The growth was poured into chilled sterile 250-ml centrifuge bottles through 4 layers of fine gauze and washed twice by centrifugation at 2000 rpm in an International No. 2 centrifuge. The partially washed growth sediments were resuspended in 0.1*N* KCl containing 0.75 mg/ml active lysozyme preparation (Nutritional Biochemicals) and placed on a rotary shaker at room temperature for 3–4 hr. The lysozyme-treated growth was further washed 10 times by centrifugation. The final spore sediments were accumulated in chilled sterile 8-oz prescription bottles with a ½-in. layer of No. 1 glass beads in the bottom, using a minimum amount of water. The lysozyme solution was sterilized by passing it through a Millipore filter, and all the water used throughout was distilled, demineralized, sterilized, and chilled.

The final spore suspension had a high degree of cleanliness as judged by its freedom from debris or any adhering sporangial material when examined via dark-phase contrast or electron microscopy.

The media used were modifications of the thermoacidurans agar (TAM); proteose peptone

0.5%, yeast extract 0.5%, glucose 0.5%, varying levels of K_2HPO_4 and $MnSO_4$, and 2% agar (broth contained none). The final reaction was adjusted to pH 6.8. Incubation, except when specified, was 5–7 days at 45°C.

B. cereus spores were produced in a liquid medium, a modification by Pelcher (1961) of the "G" medium of Church *et al.* (1954). Incubation was on a rotary shaker for 20–24 hr at 30°C. The procedure described above was used in preparing the test spore suspensions.

Thermal death rate apparatus and method. The apparatus was that described by El-Bisi and Ordal (1956a). The method was slightly modified. The heating system was brought up to the desired temperature. The reaction menstruum, 198 ml, was introduced into the reaction chamber. The chamber was loosely sealed, sterilized 15 min at 121.1°C, immediately tightened, mounted in the secondary heating oil bath, and allowed to cool to the preset equilibrium temperature. At zero time, 1 or 2 ml of the test spore suspension containing about 10^8 spores/ml were injected into the reaction chamber. Samples were withdrawn at preset intervals, chilled immediately, and plated out on the appropriate recovery medium.

The recovery medium used for both *B. subtilis* and *B. coagulans* strains was thermoacidurans agar to which 0.1% soluble starch was added, and the final reaction adjusted to pH 6.8. Incubation was made at 45°C. In the case of *B. cereus* the recovery medium was tryptone glucose extract agar (Difco), and incubation was at 30°C. Final counts were recorded after a 3-day incubation period, and all plating was in triplicate.

Analytical procedures. *Dry weight.* Appropriate samples of the spore material were pipetted into a Coors No. 2 crucible previously fired at 450°C to a constant weight. Crucibles were dried 16 hr at 105°C, cooled ½ hr in a desiccator, and weighed on a semi-microanalytical balance. The procedure of drying for an additional hour, cooling, and weighing was repeated until weight was constant.

Ash content. Dried samples were ashed in the crucibles for 16 hr at 450°C, cooled in a desiccator, and weighed on a semi-microanalytical balance. The samples were ashed for an additional hour, cooled, and weighed until weight was constant.

Cation content. The ashed spore material was dissolved in 5 ml of 0.1*N* nitric acid followed by 1–5 drops H_2O_2, and gently heated. After cooling, the dissolved contents were transferred into a 10-ml volumetric flask. The crucible was rinsed with four 1-ml portions of 0.1*N* nitric acid and made up to volume with demineralized water. This mineral extract was then used directly, or after proper dilution, for determination of the calcium, magnesium, and manganese content. One or more of the following methods were used: the emission spectrographic method as described by Malmstadt and Scholz (1955); the spectrophotometric EDTA titration procedure described by Zak *et al.* (1956); and the method of Malmstadt and Hadjiioannou (1959) for the automatic titration of calcium and magnesium. This latter method employed the Sargent-Malmstadt Spectro-Electro automatic titrator with a 650-mμ filter. In some experiments, manganese was determined with the procedure described in Standard Methods for the Examination of Water, Sewage, and Industrial Wastes (Am. Public Health Assoc., 1955).

Dipicolinic acid content. The procedure used was a slight modification of that published by Jannsen *et al.* (1958). The main modification was in the method of extracting DPA from the spore material. With our test strains it was necessary to use a more drastic treatment to provide maximum extraction of DPA. The proper sample of spore material was suspended in 0.1*N* HCl, heated 1 hr at 121° C, and cooled to room temperature; 1.5 ml of 10% trichloroacetic acid was added and thoroughly mixed; the mixture was allowed to stand 1–2 hr and centrifuged at approx. 10,000 × G; the clear supernatant was taken up and made to a volume; and the spectrophotometric determination was completed as prescribed.

RESULTS AND DISCUSSION

Effect of preheating on the spores' DPA and their subsequent thermoresistance. This study was carried out in an attempt to induce the release of DPA from the spores by exposing them to various heat treatments and to find out how such a loss of DPA would affect their subsequent thermal death rates.

Preliminary tests had demonstrated that spores stored at 3°C in distilled water lost a small but a detectable amount of DPA during storage. In this series of experiments we attempted to establish temperature-time relationships that would cause the release of appreciable amounts of DPA but have very little or no effect on spore viability. Such treated spore populations would then be subjected to a lethal temperature, and their death rates established. Although it was anticipated that other cellular components would also be released during such heat treatment, DPA was the main component monitored because of the wide speculation as to its relation to the mechanism of thermoresistance of the spore cell.

Spores of *B. subtilis* were produced on TAM agar containing 50 ppm $MnSO_4$ and 0.05% K_2HPO_4. The harvest was lysozymed and cleaned according to the procedure described previously. The spores were suspended in 2.5 m*M* phosphate buffer of pH 7 at a population level of about 4×10^9/ml. The suspension was then dispensed in 10-ml portions in sterile 50-ml Erlenmeyer flasks sealed with rubber stoppers. Flasks were placed in a constant-temperature shaker oil bath (Research Specialties Model 2156) controlled at the desired temperatures for specified periods. At preset intervals, sample flasks were taken out and subjected to DPA analysis and viable count and thermal death rate determinations at 98.5°C. in 25m*M* phosphate buffer of pH 7.

Table 1 presents data on the release of DPA and the change in viability when the spores were preheated at 45°C. Fig. 1 shows the respective thermal death rate curves (TDR) at 98.5°C. The results clearly demonstrate a limited release of DPA. Only 5.5% of the total DPA was released after a 9-day heating period at 45°C. Both viability at 45°C and thermal resistance at 98.5°C were maintained. These results indicated that a more drastic heat treatment would be required to induce significant exudation of DPA. The limited amounts of DPA released may represent a portion of cellular DPA that is present in a less stable form and is not directly involved in the mechanism of thermo-

Table 1. Effect of preheating on the release of DPA and the loss of viability of spores of *Bacillus coagulans* (M).

Preheating temperature and time[a] (hr)	% of total DPA released	Viable count ($\times 10^8$/ml)	% loss of viability
At 45°C			
0	1.40[b]	48	
10	2.97	46	
20	3.00	52	
30	4.13	47	
72	5.54	48	
216	6.87	44	
At 80°C			
0	4.4[c]	43	
3	8.0	....	
8.5	8.5	49	
At 90°C			
0	1.60[d]	45	
1.5	7.39	28	38
3	17.15	21	53

[a] Spores were heated in 2.5m*M* phosphate buffer at pH 7.0 and the level of about 40×10^7/ml.

[b] The amount of DPA found in the supernatant of unheated control spores stored 30 days at 3°C. The DPA level in these spores was approx 8%.

[c] Spores used were previously preheated 20 hr at 45°C. This number includes the DPA released during 3°C storage (1.4%) and that released during the 45°C heat treatment (3.0%).

[d] The amount of DPA found in the supernatant of unheated control spores stored at 3°C.

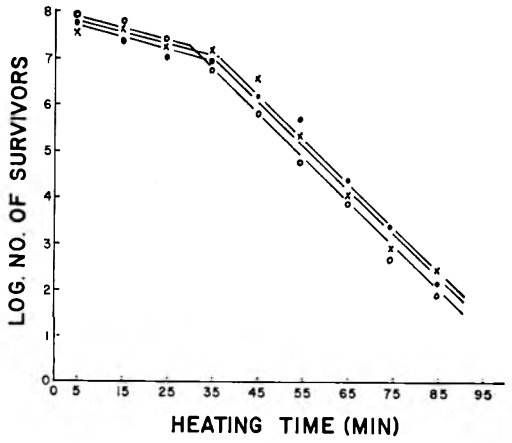

Fig. 1. Thermal death rate curves of spores of *Bacillus subtilis* preheated at 45°C. Death rates were determined in 2.5m*M* phosphate buffer, pH 7, at 98.5°C. ○, unheated control; ●, spores preheated for 20 hr; ×, spores preheated 30 hr.

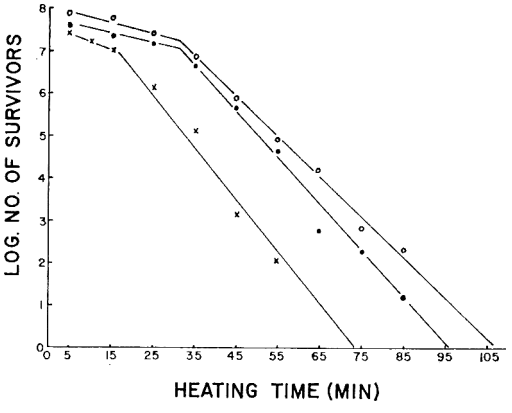

Fig. 2. Thermal death rate curves of spores of *Bacillus subtilis* preheated at 80°C. Death rates were determined in 2.5m*M* phosphate buffer, pH 7, at 98.5°C. ○, unheated control; ●, spores preheated 3 hr; ×, spores preheated 8.5 hr.

resistance of the spore cell, or it may result from a few germinated spores.

To induce a greater release of DPA, similar experiments were carried out at 80 and 90°C. Table 1 also provides data on the release of DPA and the changes in viability at 80°C. Fig. 2 illustrates the respective TDR curves at 98.5°C. The viability of the spores remained unchanged throughout the 8.5-hr heating period. The amount of DPA released was still limited, being only 3.6 and 4.1% at the end of 3 and 8.5 hr of preheating. Spores treated in this manner exhibited some loss in thermoresistance as the preheating period was extended, but such a loss was not proportional to the loss in DPA.

Table 1 and Fig. 3 present the data obtained when spores were preheated at 90°C. The release of DPA was increased by this treatment, but continued exposure to this temperature also reduced the viability of the spore suspension. The loss in heat resistance was related to the length of the preheating treatment but again did not correlate with the amount of DPA released.

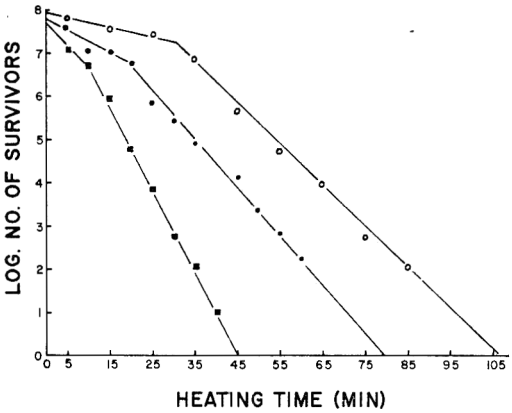

Fig. 3. Thermal death rate curves of spores of *Bacillus subtilis* preheated at 90°C. Death rates were determined in 2.5m*M* phosphate buffer, pH 7, at 98.5°C. ○, unheated control; ●, spores preheated 1.5 hr; ■, spores preheated 3 hr.

A subsequent experiment was undertaken to characterize more fully the changes in a spore suspension during the preheating period. Spore suspensions were heated at 80, 85, and 90°C, sampled at intervals, and analyzed for survivals, and ninhydrin-positive material (NPM) and DPA in the supernatant. As previously demonstrated, very little DPA was released when the spores were held at 80°C. This was also true of the NPM. As the heating temperature was increased, there was a marked increase in the release of both DPA and NPM (Fig. 4). However, the increased rate of release of NPM with temperature was greater than

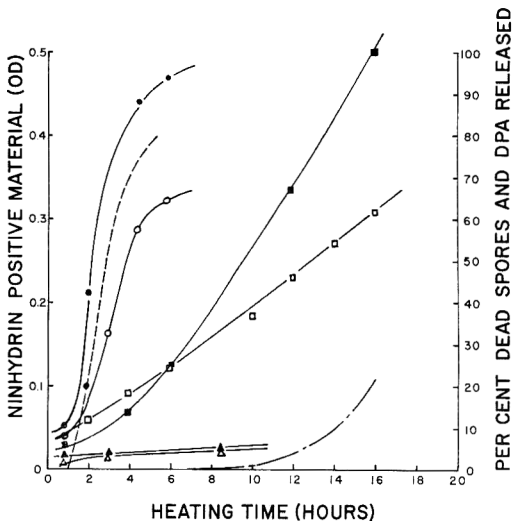

Fig. 4. Release of cellular components and loss of viability of spores of *Bacillus subtilis* during heating in 2.5m*M* phosphate buffer at pH 7. Spores heated at 80°C: ▲, ninhydrin-positive material; △, DPA; no apparent loss of viability. Spores heated at 85°C: ■, ninhydrin-positive material; □, DPA; — - —, percent dead spores. Spores heated at 90°C: ●, ninhydrin-positive material; o, DPA; - - - -, percent dead spores.

that of DPA and was more closely related to the rate of death. It is felt that NPM could be related to or similar to those nitrogenous components released from the spore wall during germination of *Bacillus* species as reported by Powell (1957). The release of such material could be the initial change that sets the stage for further loss of cellular components, and possibly the onset of the death reaction.

Thermal death and the release of DPA of spores of varied thermoresistance. Three different spore suspensions were prepared of *B. coagulans* (thermoacidurans) of different degrees of thermal resistance. The phosphate level in the sporulation medium was the determinant factor responsible for the resultant variation in thermoresistance.

Crop A, of the highest resistance, was produced in TAM agar containing 0.05% K_2HPO_4; and Crop C, of the lowest resistance, was produced in the presence of 0.5% K_2HPO_4. All media contained 1 ppm $MnSO_4$.

Both thermal death rate and the DPA exudate were determined simultaneously for each of the three crops at 95°C in 25m*M* phosphate buffer at pH 7.

Fig. 5 illustrates the results. It is apparent that death followed different kinetics from those followed by the release of DPA. While the former seems to conform to a first-order reaction, the latter more closely follows that of a zero-order reaction. However, higher death rates were associated with higher rates of DPA release.

Release of DPA and divalent cations during thermal death in varied heating menstrua. This experiment was designed primarily to establish the changes in the spore material during thermal death, and to explore the relationship, if any, between certain changes and the death reaction. The indices of change selected for this purpose were dry weight, calcium, magnesium, manganese, and DPA.

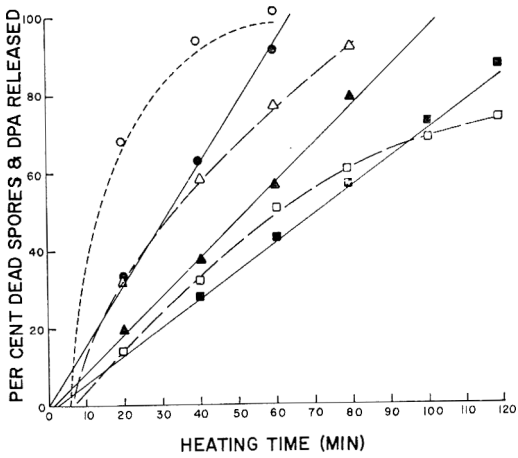

Fig. 5. Release of DPA during thermal destruction of spores of varied thermoresistance. Spores of *Bacillus coagulans* (thermoacidurans) were produced on agar media containing various levels of phosphate; Crop A, 0.05%; Crop B, no added phosphate; Crop C, 0.5%. Spores were heated in 2.5m*M* phosphate buffer at pH 7. Crop A: □, dead spores; ■, DPA released. Crop B: △, dead spores; ▲, DPA released. Crop C: o, dead spores; •, DPA released.

A spore crop of *B. subtilis* was produced on TAM agar containing 0.05% K_2HPO_4 and 20 ppm $MnSO_4$. The final suspension contained more than 10^{10} spores/ml. Six 25-ml samples of this suspension were pipetted into six 50-ml volumetric flasks. The first five flasks were diluted with similar volumes of sterile demineralized water, and the sixth flask with 20m*M* glycylglycine to give a final concentration of 10m*M* GG. All flasks were heated in a pre-equilibrated American Sterilizer autoclave equipped with Cyclomatic and Isotherm Controls set at 98°C, sampled at preset intervals, and analyzed for survivors, dry weight, ash, calcium, magnesium, manganese and DPA. It was recognized that, because of such a heavy suspension and the large size of sample, sampling errors would be greater, from varied heat transfer during heating and cooling and uneven dispersion of spores. To counterbalance such errors, triple analyses were made on each sample, the total spore material, the supernatant, and the spore sediment. To overcome the problem of extremely low levels, especially of the divalent cations, in the supernatant, internal standard levels of the test constituents were added to all supernatant samples prior to analyses. Such built-in standards also served as monitors of the analytical results. The water samples were to compare among each other and establish the trend in the various reaction rates, and the sample heated in GG was to demonstrate the effect of such a death-accelerating agent upon these rates of various reactions.

Table 2 presents the data obtained. The average composition was: calcium 2.57, magnesium 0.208, manganese 0.048, and DPA 7.75% of the total dry weight of spores. These values were used as the reference for computing the data illustrated in Fig. 6. Death progressed at the highest rate, suggesting that death precedes other chemical changes. Manganese was exuded at a much greater rate than calcium. Magnesium was released at the slowest rate. The rate of manganese exudation was the closest to that of death during the initial heating stage. While death and the exudation of cations seem to have followed a first-order reaction

Table 2. Behavior of dipicolinic acid and divalent cations during thermal destruction of spores of *B. coagulans* (M) at 98°C in varied heating menstrua.

Sample heated		Viable count ($\times 10^{10}$/ml)	Dry weights (mg/ml)	Composition in % of original dry weight of spores [a]			
				Calcium	Magnesium	Manganese	DPA
0 min	Total	1.2	5.16	2.82	0.23	0.05	7.75
in water	Supernatant	....	0.108	0.068	0.001	0.005	0.23
	Sediment	....	4.356	2.68	0.204	0.025	7.34
5 min	Total	0.71	3.98	2.54	0.22	0.05	7.84
in water	Supernatant	....	0.196	0.101	0.006	0.019	0.72
	Sediment	....	3.680	3.11	0.291	0.048	7.74
10 min	Total	0.63	4.30	2.45	0.21	0.055	7.34
in water	Supernatant	....	0.284	0.260	0.008	0.022	1.37
	Sediment	....	3.547	2.46	0.257	0.039	6.81
25 min	Total	0.26	4.22	2.46	0.257	0.03	7.80
in water	Supernatant	....	0.344	0.82	0.006	0.020	3.11
	Sediment	....	3.534	2.02	0.215	0.036	4.93
65 min	Total	0.00316	4.36	2.64	0.21	0.05	7.91
in water	Supernatant	....	0.702	1.15	0.028	0.032	7.34
	Sediment	....	3.144	0.972	0.199	0.016	0.65
25 min	Total	0.000526	4.32	2.52	0.22	0.055	7.89
in M/100	Supernatant	....	0.596	1.29	0.031	0.047	5.97
glycylglycine	Sediment	....	3.314	1.49	0.222	0.023	2.06
Av. spore composition (% dry weight)				2.57	0.208	0.048	7.75

[a] Cations determined by emission spectrograph.

rate, DPA seems to have followed a zero-order reaction rate. The accelerated death in the presence of GG was associated with an accelerated exudation of manganese, DPA, and calcium. Magnesium was less affected. The 25-min survival and the exudate levels in GG were nearly equivalent to those in the 65-min water sample.

Table 3 compares the released components on the basis of percent dry weight of total cellular exudate. The unidentified material in the exudate made up over 80% of its dry weight during the initial heating stage, and became progressively smaller until it reached about 45%, at the end of the experiment. Part of such material could be the ninhydrin-positive type previously demonstrated. Analogous to the germination exudate reported by Powell (1957), this material could be made up primarily of certain spore wall nitrogenous components the release of which precedes that of the other cellular components.

Kinetics of calcium and DPA release during thermal death. Spores were exposed to lethal temperatures, sampled at close intervals, and analyzed for death as well as calcium and DPA release. A controlled-temperature oil shaker bath was preset at the desired temperature. Sterile 50-ml Erlenmeyer flasks, each containing 3.5 ml of 10mM phosphate buffer of pH 7, were placed on the shaker and allowed to equilibrate. Each flask was then inoculated with 3.5 ml heavy spore suspension, resulting in a final phosphate buffer concentration of 5mM as the heating menstruum. At pre-set time intervals one flask was removed and immediately chilled in an ice bath, one ml was removed for a survival count, and the rest was transferred quantitatively into a chilled 50-ml thermostable plastic centrifuge tube and centrifuged at approx 10,000 $\times$G. The supernatant was transferred into a 10-ml volumetric flask and made up to volume. The pellet was acidified by adding 10-ml 0.1N HCl and autoclaving for 1 hr. The tube was then recentrifuged and the supernatant transferred into a 25-ml volumetric flask and made up to volume. Both contents

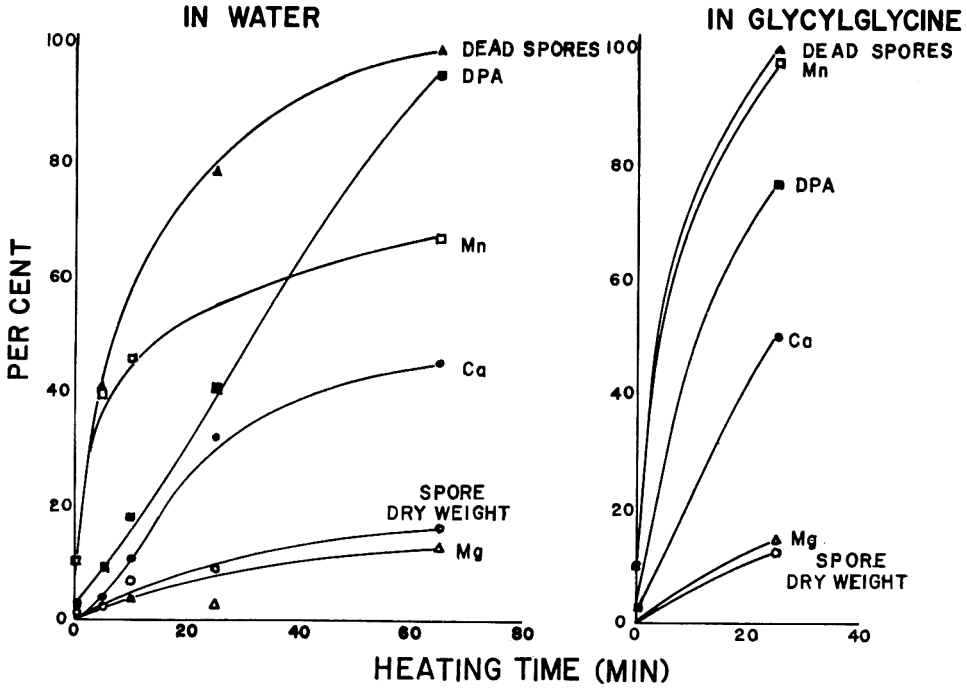

Fig. 6. Rate of release of cellular components from spores of *Bacillus subtilis* during thermal destruction in two heating menstrua at 98°C.

in the two volumetric flasks were analyzed for calcium and DPA; the former representing the exudate and the latter the residue in heated spores. The test organisms used were *B. coagulans* (thermoacidurans) heated at 96 and 99°C, and *B. cereus* heated at 86°C. Spores of the former strain were produced on the TAM agar with 0.05% K_2HPO_4 and 1 ppm $MnSO_4$, whereas the latter were produced in the modified G medium.

Figs. 7 and 8 illustrate the results for *B. coagulans* (thermoacidurans). The death reaction progressed at a higher rate than that for the release of either DPA or calcium. Initially, calcium was released more rapidly than DPA, but the rate of release of both substances was reduced as heating continued until a fixed amount remained in the spores. Such behavior led to a higher molar ration of Ca/DPA in the exudate during the initial heating period. This ratio dropped rapidly and eventually reached a constant value for the remainder of the heating period (Table 4). The behavior pattern was similar at the two test heating temperatures. Both the initial and intermediate stages are clearly represented at

Table 3. Percent composition of exudate during the thermal destruction of spores of *B. coagulans* (M) at 98°C in varied heating menstrua.

Sample heated	Percent of spore's dry weight exuded	Percent composition of dry weight of exudate				
		Calcium	Magnesium	Manganese	DPA	Unidentified material
0 min in water	2.09	2.69	0.038	0.185	10.84	86.26
5 min in water	2.43	1.70	0.097	0.322	14.70	83.18
10 min in water	6.60	3.25	0.105	0.281	20.78	75.58
25 min in water	8.12	8.31	0.060	0.208	38.08	53.34
65 min in water	16.10	5.95	0.146	0.166	45.58	48.16
25 min in glycylglycine	13.79	7.78	0.189	0.285	43.30	44.45

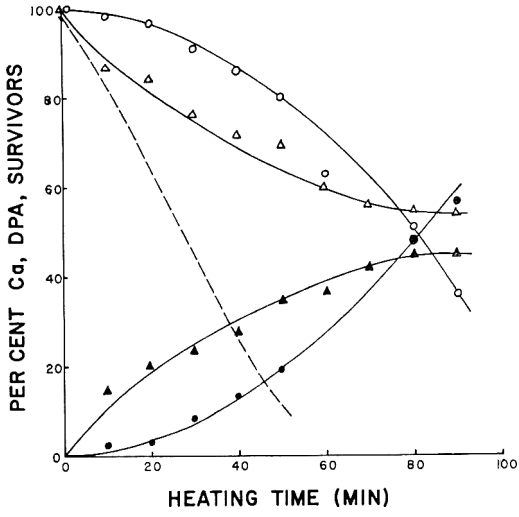

Fig. 7. Chemical changes during thermal destruction of spores of *Bacillus coagulans* (thermoacidurans) at 96°C. Spores were heated in 5m*M* phosphate buffer at pH 7. o, DPA in heated spores; ●, DPA in supernatant; △, calcium in heated spores; ▲ calcium in supernatant, – – – –, percent survivors.

the lower heating temperature, and the late or final stage is clearly represented at the higher temperature. A similar pattern was followed when *B. cereus* spores were heated at 86°C (Fig. 9 and Table 4).

It has been adequately demonstrated that the thermal death reaction is associated with the release of dipicolinic acid and certain divalent cations such as calcium and manganese. Factors that accelerated the death reaction also accelerated the release of the above cellular components. Whether such an exudate is the cause or the effect of the thermal death of the spore cell remains difficult to determine. From the previously illustrated kinetics of both phenomena, it appears that death precedes the release of DPA and divalent cations. The possibility exists, however, that in the latter case we may be dealing with a two-step reaction. First, the disruption within the spore cell of a certain critical stereostructure that relies on such components as DPA and the divalent cations to act as the cementing material; and second, the release of such widely varied DPA-divalent cation molecular combinations from the disrupted particulate type structure into the surrounding menstruum. This latter step could in itself be composed of two successive steps instead

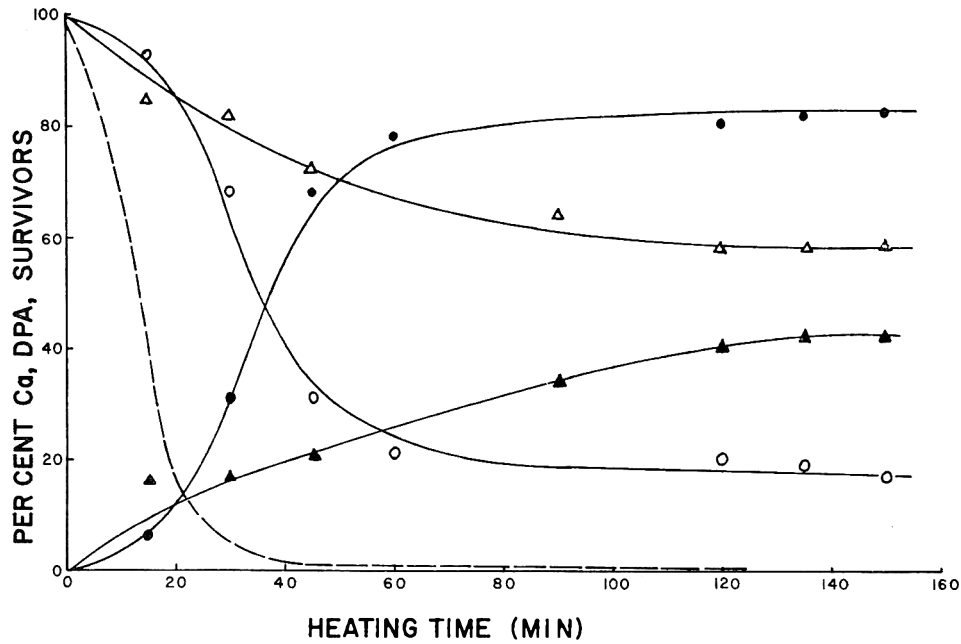

Fig. 8. Chemical changes during thermal destruction of spores of *Bacillus coagulans* (thermoacidurans) at 99°C. Spores were heated in 5m*M* phosphate buffer at pH 7.0. o, DPA in heated spores; ●, DPA in supernatant; △, calcium in heated spores; ▲, calcium in supernatant; — — — —, percent survivors.

Table 4. Molar ratios of calcium to dipicolinic acid as retained and released by the spores during their thermal destruction.

Heating time (min)	*B. coagulans* (thermoacidurans)[a]		*B. cereus*[b]	
	Retained	Released	Retained	Released
5	....	....	0.7	1.1
10	....	....	0.7	1.1
15	1.1	3.5	0.7	0.9
20	....	....	0.7	0.8
25	....	....	....	....
30	1.6	0.70	0.8	0.7
35	....	....	0.8	0.7
40	....	....	0.8	0.7
45	4.3	0.47	....	....
50	....	....	0.8	0.7
60	7.6	0.59	0.8	0.7
70	....	....	....	....
90	2.39	0.52	....	....
105	2.51	0.46	....	....
120	3.03	0.51	....	....
135	3.14	0.54	....	....
150	3.60	0.56	....	....

[a] Heated in *M*/200 phosphate buffer at pH 7 at 99°C.
[b] Heated in *M*/200 phosphate buffer at pH 7 at 86°C.

of one; first, the release or freeing of such small molecular fragments (DPA-cation) from the disrupted structure, and second, the exudation of such free fragments into the surrounding menstruum. Such sequential-type reactions or events are extremely difficult, if not impossible, to resolve and evaluate. It is highly probable that each step in such a complex chain of events is governed by a single major or multiple major factors, and that the final exudate is the over-all net result of all such interfering factors.

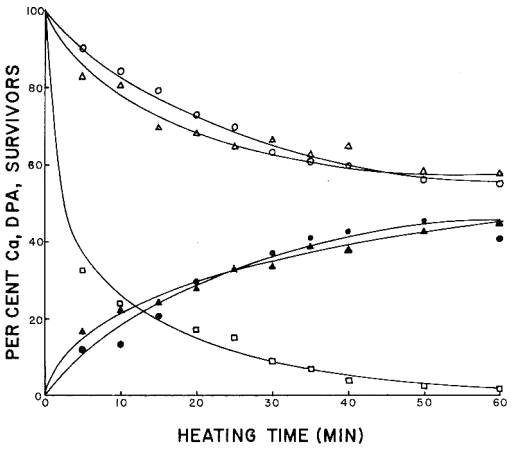

Fig. 9. Chemical changes during thermal destruction of spores of *Bacillus cereus* at 86°C. Spores were heated in 5m*M* phosphate buffer at pH 7.0. o, DPA in heated spores; ●, DPA in supernatant; △, calcium in heated spores; ▲, calcium in supernatant; □, percent survivors.

Another major difficulty one encounters in correlating the death reaction with such exudates is the possibility that such cellular components as DPA, divalent cations, and maybe others could possibly exist in multiple cellular structures, one of which may be directly related to or exercise control upon the death reaction, while the others do not and serve entirely independent functions. All forms are liable to the thermal degradative effect, and the final exudate is again the indirect over-all result of such a multitude of behaviors. Therefore, the measured rate of release of a certain cellular component in the heating menstruum need not be representative of its critical form associated with the mechanism of thermoresistance.

A third and apparently insoluble problem would be the possibility that a certain cellular component is being exuded from more than one cellular form: a surviving cell, a dying cell, and a cell already dead. So far, it is impossible to differentiate between such various exudate fractions in a heated spore population, and hence impossible to establish a direct relationship, if any, with the thermal death reaction.

However, as has been occasionally shown, the level of certain cations such as calcium and/or manganese as well as certain molar ratios such as cations/DPA in the spore cell may still serve as indices of its thermoresistance.

It is noticed in Figs. 1, 2, and 3 that although the death rates assumed what appears to be the first-order kinetics, an initial lag or shoulder was encountered in all cases. Such behavior has often been encountered by many workers in the field and often attributed to inherent multicellular groupings, clumping, heat activation of surviving cells, carry-over of antigermination or antigrowth principle(s) . . . etc. (El-Bisi and Ordal 1956). Two further observations, which may be related to this

phenomenon are noteworthy: (a) the initial lag or shoulder diminished with the increase of the preheating temperature and time (Figs. 1, 3,) and (b) there had been what appeared to be a relatively higher rate of cation and DPA exudation during the intial heating period (Figs. 7, 8, 9). These observations suggest that such initial lags or shoulders in thermal death rate curves could be due (at least in part) to an enriched protective steareostructure involving DPA and enhanced by higher levels of divalent cations. Such structure is independent of and external to the site of death, and while being degraded during the initial heating stage it is consuming a significant part of thermal energy and hence a lower initial death rate.

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Effect of Processing Temperature on Pigments and Color of Spinach[a]

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SUMMARY

A method was developed for estimation of chlorophylls a and b, pheophytins a and b, lutein, and carotene in processed spinach. The method, developed primarily for colorimetric studies, involves extraction of the spinach with acetone and chromatographic separation on a sugar-starch (70:30) column. Equations were developed for spectrophotometric estimation of the amount of each pigment in the eluate from the column. The method gave recovery values of 95-98% for a wide range of pigment mixtures, and a coefficient of variation of approximately 1.5% for reproducibility on the same extract.

Fresh spinach was blanched, pureed, packed in glass thermal-death-time tubes and processed in an oil bath at 240, 250, 260, 270, and 280°F for a process value equal to Fo 4.9. Color measurements indicated a difference of 4.1 units between the controls and the samples processed at 280°F, and 11.6 units between the controls and the samples processed at 240°F. The pigment changes indicated a progressively smaller change in chlorophylls a and b to pheophytins a and b as the processing temperature was raised. Chlorophyll a was degraded more rapidly than chlorophyll b, and the ratio of the two changed from 1.55 for the samples processed at 280°F, to 0.92 for the samples at 240°F. Some degradation of lutein was observed, particularly at the lower processing temperatures, whereas carotene was unchanged. The pigment-free tissues also showed more change in color at the lower temperatures, but the contribution of the pigment-free tissues and the degradation of lutein to the over-all color change was very small. The major reasons for the change in color of the spinach puree upon processing were, first, the degradation of chlorophyll a to pheophytin a, and second, the degradation of chlorophyll b to pheophytin b.

INTRODUCTION

The change in color of foods containing chlorophyll from a bright green to an olive green on processing has been of concern to food processors since the introduction of thermal processing. The color change has been attributed to the conversion of chlorophyll to pheophytin in peas (Gold and Weckel, 1959, Mackinney and Weast, 1940) and green beans (Westcott *et al.*, 1955). Since the same reaction was described as the reason for the color change in frozen peas (Mackinney and Weast, 1940, Campbell, 1937, 1950) and dehydrated spinach (Dutton *et al.*, 1943), it is probably a general reaction for most products containing chlorophyll.

The kinetics of conversion of chlorophyll to pheophytin at different temperatures were studied in aqueous acetone extracts by Mackinney and Joslyn (1941). They concluded that the rate was first-order with respect to both chlorophyll and acid. Gold and Weckel (1959) also studied the kinetics of degradation of chlorophyll in peas under the conditions necessary for thermal preservation. They concluded that the conversion of chlorophyll to pheophytin followed pseudo-first-order kinetics, and were able to predict that raising the temperature of processing with an equivalent Fo value would decrease

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the degradation of chlorophyll from 74.1% at 240°F (pH = 6) to 1.09% at 280°F. The calculation of 1.09% degradation was, of course, based on the assumption of instantaneous heating and cooling. This might be difficult to approach in practice because, as the authors pointed out, 50% of the chlorophyll might be degraded during the retort "come-up" time. Even with a Votator operated at 280°F (Kiratsous *et al.*, 1961), the heating and cooling curves could contribute up to 65% of the Fo value. However, the contribution of the heating and cooling curves could be included in the calculation of the Fo value.

It is reasonable to assume that raising the temperature of processing while maintaining an equivalent Fo value would result in a different retention of chlorophyll because the kinetics of chlorophyll degradation are different from the kinetics of the death of microorganisms. Most sterilization values for processed foods are calculated with a "z" value of the order of 18 (Ball and Olson, 1957), whereas most chemical reactions exhibit a "z" value of the order of 60°F (Adams and Yawger, 1961). Fortunately, in this case, the heat requirements to achieve the bacteriological aims are such that more chlorophyll is retained at the higher temperatures.

This work was undertaken to establish quantitatively the changes in color and pigment content in spinach as the temperature of processing was raised with an equivalent Fo value.

MATERIALS AND METHODS

Fresh spinach from a local wholesale market was washed, trimmed, and blanched for 3 min in boiling water. The spinach was chilled immediately in ice water, and then held on a screen for 20 min to drain off excess water. The blanched spinach was then pureed in a Fitzpatrick mill, using a coarse screen and then a fine screen (no. 40) for the second passage through the mill. Immediately after comminution the pureed spinach was deaerated to remove the oxygen. The deaeration was accomplished by allowing the puree to pass slowly from a separatory funnel into a desiccator maintained at a vacuum of 28 mm Hg. After deaeration, the puree was stored at 38°F until processed. The color changes in the puree were negligible even up to 14 days at 38°F.

The moisture content of every batch of strained spinach ranged from 92.5 to 94.5%.

For retort processing, baby-food jars (201 × 210) were chosen as convenient containers. To determine the heat penetration rate, a bakelite-stem thermocouple was fixed to the center of the jar cover such that it would penetrate to the center of the jar. After heating in a water bath to the required initial temperature with careful stirring to avoid the incorporation of air bubbles, the puree was filled into the jars and sealed. The jars were processed under water in a retort with automatic controls, and temperatures were taken every 3 min. The sterilization values were calculated according to the General Method, using a "z" value of 18 (Bigelow *et al.*, 1920; Ball and Olsen, 1957).

For processing in an oil bath, glass thermal-death-time tubes (TDT tubes) were chosen because the sterilization value could be measured at the higher temperatures more accurately with this method. Tubes were prepared from Pyrex tubing (7 mm ID, 1 mm thickness, 15 cm in length), and filled with puree using a glass tube of smaller diameter under air pressure. Thermocouple wires were fixed in the center of selected TDT tubes and attached to a temperature recorder. The tubes were conditioned in a water bath to adjust the initial temperature and immersed in an oil bath. Temperature readings were taken at 15-sec intervals during the heating and cooling cycles. The process values (Fo) were calculated by the General Method.

Since the determination of heat penetration data was for the purpose of designing equivalent thermal-processing conditions for both the retort and oil bath at several temperatures, the data were computed and expressed as the relation between heating time and Fo value. According to the recommendations of the National Canners Association (N.C.A., 1955), strained spinach in small baby-food jars (201 × 210, 3½ oz) requires an Fo value equal to 4.9. Table 1 shows the times and other conditions necessary to achieve this value for each processing condition.

The color determinations on the puree were made with a Colormaster Differential Colorimeter (Manufacturers Engineering and Equipment Corp., Hatboro, Penn.) equipped with a 10× multiplier and a small-area aperture. A gray tile (G = 5.52, R = 5.49, B = 6.22) was used to standardize the instrument. Color data were also taken with a Gardner Color and Color-Difference Meter (Gardner Laboratory, Inc., Bethesda 14, Md.), manual model, equipped with a small-area aperture. A green tile (L = 19.2, a = −12.2, b = 0.2) was used to standardize the instrument. The color data

Table 1. Processing conditions for spinach for conditions equivalent to Fo = 4.9.

Method	Processing temp (°F)	Initial temp (°F)	Processing time (min)	Cooling time (min)
Retort (baby-food jars, 3½ oz)	240	130	37	15
	250	130	22	15
Oil bath (TDT tubes)	240	90	19.5	Immediate
	250	90	6.7	cooling
	260	90	3.17	in
	270	90	1.95	ice
	280	90	1.60	water

from the Gardner instrument were calculated as the "–a/b" index of hue in order to make the data compatible with the nomographs calculated for peas by Gold and Weckel (1959). The color data from the Colormaster were converted to the Adams Coordinate System and used to calculate a color difference (Delta E) between the control and the processed sample. The color difference was calculated in NBS units by the equation:

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

The pigment-free residues were obtained by blending a 10-g sample of spinach puree for 2 min with 120 ml of 85% acetone in water in an Omnimixer. After filtering and washing with pure acetone, the residue was air-dried and stored until the color measurements could be performed. The pigment-free residues were moistened with acetone, and measured on a Colormaster colorimeter standardized against a white tile (G = 84.97, R = 84.44, B = 83.74). Delta E color differences between the control and the processed samples were calculated in the same manner as for the puree.

Development of an analytical method for pigment determinations. A variety of methods have been suggested for determination of the pigments in chlorophyllaceous plant materials. They range in complexity from a relatively simple extraction with 85% acetone (Petering *et al.*, 1949) to a paper chromatographic method (Hager, 1957) for separating all the pigment constituents. The present work sought a method for estimation of the principal pigments present in processed spinach for studies on color changes due to processing.

In preliminary work, several of the better known methods were examined for their suitability for processed spinach. The determination of chlorophylls has been studied extensively by Zscheile (Zscheile and Comar, 1941; Zscheile *et al.*, 1942), and their equations were adopted in the A.O.A.C. (1960) method. Aronoff (1953) modified the equations of Comar and Zscheile (1942) to include two additional equations to solve for the concentration of pheophytins a and b. Sweeney and Martin (1958) modified the formulae developed by Comar and Zscheile for use with chlorophylls in acetone solution and also developed equations to estimate the relative amount of chlorophyll present in extracts of broccoli after various cooking procedures. Mackinney and Weast (1940) and Dietrich (1958) developed equations for estimating the concentration of chlorophyll and percent conversion to pheophytin. The methods, involving relative conversion of chlorophylls to pheophytins without estimating the amount of chlorophylls a and b and pheophytins a and b, were unsuitable for studies involving an explanation of color changes upon processing, because the colors of the four pigments are different. Chlorophyll a has an intense blue-green color, which changes to the gray color of pheophytin a. Chlorophyll b has a yellow-green color, which changes to the olive-green color of pheophytin b.

The A.O.A.C. method and Sweeney and Martin's modified method were quite suitable for the determination of total chlorophyll in processed spinach extracts, and were found to agree within 0.5% when little or no pheophytin was present. Calculations for total chlorophyll and pheophytin in spinach extracts averaged 9.1% higher with the Sweeney and Martin equation for absorbance at 558 mμ than with the Sweeney and Martin equation for total chlorophyll. A comparison of the two methods using the actual intersection point, which averaged 553 mμ in this laboratory, gave results that agreed within 2.7%. This serves to emphasize the recommendation of Sweeney and Martin (1958) that each analyst develop his own constants. The equations developed by Aronoff gave obviously incorrect values with some extracts of processed spinach, so this method was not applicable to extracts with a high proportion of pheophytins.

The data from the preliminary work indicated that none of the existing methods would be entirely suitable for color studies, so an attempt was made to develop a suitable method. However, since this work was completed, Vernon (1960) has published a method for estimating chlorophylls

a and b and pheophytins a and b. Sweeney and Martin (1961) also published a modified method for estimating chlorophylls a and b that probably could be adapted to the estimation of pheophytins a and b.

It was evident that a method designed to aid in an understanding of color changes due to processing would have to be capable of estimating each pigment that might be involved in the color change. With spinach, this would include chlorophylls a and b, pheophytins a and b, and the two major carotenoids, carotene and lutein. A chromatographic approach seemed to be most promising for this goal.

A number of combinations of sugar, starch, and talc were investigated in combination with many combinations of petroleum ether, acetone, benzene, ethanol, and methanol. A column composed of 70% powdered sugar and 30% corn starch used with 2% and 3% acetone in petroleum ether was found to be a suitable combination for separation of the six pigments.

Identification of pigments on chromatogram. Purified preparations of pheophytins a and b were prepared from spinach extracts by treatment with oxalic acid and repeated chromatography on sugar-starch columns. Purified preparations of the carotenoids were prepared from spinach extracts by refluxing an acetone solution with barium hydroxide (Petering *et al.*, 1940) and repeated chromatography on sugar-starch columns. The purified pigments were evaporated to dryness and redissolved in the appropriate solvent. Spectral data for identification purposes are presented in Table 2.

The pigments in zones 3 and 5 were respectively identified as pheophytin b and a. The pigments in zones 2 and 4 were respectively violaxanthin and lutein. The pigment in zone 1 was probably composed of several carotenoids, but since they were present in small amounts and probably would have little effect on the color of the spinach puree, no attempts were made to identify them further. The pigment in the yellow solution that was not absorbed by the column was mainly beta-carotene. According to Wall and Kelly (1943) and Kemmerer and Fraps (1943), this solution is likely to include alpha-carotene and neo-beta-carotene as well as beta-carotene. However, the amount of alpha-carotene in spinach is very low as compared with beta-carotene.

Determination of specific absorption coefficients for pheophytins a and b. Pheophytin a was isolated chromatographically from an oxalic-acid-treated extract of spinach. After five passages through a sugar-starch (70:30) column, the purified pigment was recovered in petroleum ether. Upon evaporation the pheophytin a precipitated out and was redissolved in acetone. After precipitation from acetone the pigment was dried, weighed, and redissolved in petroleum ether containing 2% acetone. Pheophytin b was handled in the same manner except that the purified pigment was redissolved in petroleum ether containing 3% acetone.

Absorption curves for the above preparations were determined with a Beckman DU spectro-

Table 2. Identity of pigments isolated from chromatograms of petroleum ether extracts of spinach with oxalic acid.

Zone[a]	Color	Solvent	Absorption maximum (mμ) Observed	Reported	Pigment
1	Light yellow	Chloroform	405, 428	.....	Unidentified
2	Dark yellow	Benzene	425, 450 483	425, 449 482	Violaxanthin[b]
3	Olive green	Ethyl ether	433, 523 599, 655	433, 523 599, 655	Pheophytin b[c]
4	Yellow	Benzene	430, 456 487	430, 456 487	Lutein[d]
5	Gray	Ethyl ether	409, 506 532, 608 667.5	409, 506 532, 608 667.5	Pheophytin a[e]
6	Yellow (not absorbed on column)	Petroleum ether	447	447	Carotene[f]

[a] In order of decreasing absorbance on a sugar-starch (70:30) column.
[b] Curl and Bailey, 1954.
[c] Zscheile and Comar, 1941.
[d] Curl, 1953.
[e] Aronoff, 1950.
[f] O'Connor *et al.*, 1946.

photometer. The specific absorption coefficients for the two pigments were:

Pheophytin a: 56.8 L/g cm at 667.5 mμ in 2% acetone in petroleum ether (b.p. 36–54°C)
Pheophytin b: 31.8 L/g cm at 665.0 mμ in 3% acetone in petroleum ether (b.p. 36–54°C)

These data were necessary to derive the equations for content of pheophytins a and b as presented in the proposed method.

The purity of the isolated pheophytins a and b was checked by determining their specific absorption coefficients in ethyl ether. The values obtained were respectively 59.1 and 36.0 L/g cm for pheophytins a and b, which compared favorably with those (59.5 and 37.5) reported by Zscheile and Comar (1941).

Analytical procedure. The procedure followed in extracting the pigments from the spinach tissue was very similar to the A.O.A.C. (1960) method except that petroleum ether was used instead of ethyl ether in order that the final pigment solution would be ready for chromatographic separation.

A 10-g sample of spinach puree was mixed for 2 min with 120 ml of 85% acetone in water and about 0.1 g of calcium carbonate in an Omnimixer (Ivan Servall, Inc., Norwalk, Conn.). The mixture was filtered in a Buchner funnel, and washed free of pigment with 85% acetone and finally with a few ml of pure acetone. The combined extract and washings were transferred to petroleum ether with a small modification in the A.O.A.C. method, based on the suggestion by Hager (1957). A 10% sodium chloride solution was used instead of water to "salt-out" the pigment from the acetone-water layer to the petroleum ether layer. The pigment solution in petroleum ether was made up to 200 ml, dried over sodium sulphate, and used in the chromatographic separation.

The chromatographic columns were prepared from a mixture of 70% confectioners sugar (6×) and 30% corn starch (Melojel, obtained through the courtesy of the National Starch and Chemical Corp., New York). The absorbent mixture was dried for 12 hr at 100°C before being packed into a 15 × 300-mm column. Fifteen g of absorbent were made into a slurry with petroleum ether, poured into the tube, and concentrated under air pressure. A small disc of filter paper was added, and then a 0.5-cm layer of anhydrous sodium sulphate. The excess petroleum ether was removed with air pressure, and 10 ml of the pigment extract were added. After the pigment was absorbed on the column, 15 ml of petroleum ether was added to wash the carotene from the column. The pheophytin a was eluted with 35 ml of 2% acetone in petroleum ether. The pheophytin b was eluted together with lutein with 45 ml of 3% acetone in petroleum ether. The chlorophyll was eluted with 15 ml of acetone. Each eluate was collected and made up to volume.

The calculations for pigment content were made after absorption with appropriate dilution was measured with a Beckman DU spectrophotometer.

The calculations for total chlorophyll as well as chlorophylls a and b were based on the equations of Comar and Zscheile (1942), adopted in the A.O.A.C. method. A correction factor of 1.06 was used to modify the equations for use in the acetone solution from the adsorption column. The modified equations were as follows:

Total chlorophyll (mg/L) = 7.55(A660) + 17.8 (A642.5)
Chlorophyll a (mg/L) = 10.5(A660 − 0.824 (A642.5)
Chlorophyll b (mg/L) = 18.6(A642.5 − 2.98 (A660)

Pheophytin a was calculated by the equation:

Pheophytin a (mg/L) = 17.6(A667.5)

Pheophytin b and lutein were determined in the same solution. Since lutein had no absorbance at 655 mμ (Zscheile *et al.*, 1942), the concentration of pheophytin b was calculated by assuming it as the only component of the solution absorbing at 655 mμ.

Pheophytin b (mg/L) = 31.4(A655)

Lutein was calculated by developing an equation for the absorbance of lutein at 472 mμ corrected for the absorbance of pheophytin b at the same wavelength. Lutein has a specific absorption coefficient of 231 L per g cm at 477.5 mμ in ethanol (Zscheile *et al.*, 1942). The absorption coefficient of lutein in 3% acetone in petroleum ether was determined as 250 L per g cm at 472 mμ, by comparison with a similar solution in ethanol. Since pheophytin b has an absorption coefficient of 3.3 at the same wavelength, the following equation could be derived.

Lutein (mg/L) = 4.00(A472) − 0.415(A655)

The calculation for carotene was based on the observation that beta-carotene has a specific absorption coefficient of 245 L per g cm at 447 mμ in petroleum ether (O'Connor *et al.*, 1946):

Carotene (mg/L) = 4.08(A447)

No equation to estimate the concentration of violaxanthin was developed, because it was present in relatively small amounts and could not be expected to have much influence on the color change from processing.

Recovery and reproducibility studies. The accuracy of the method was determined by the recovery of each component in a pigment extract.

Table 3. Recovery of pigments in synthetic mixtures.

Pigment	No. of samples	Range of pigment added (mg/L)	Mean recovery	Coefficient variation recovery (%)
Solution A				
Pheophytin a	20	3.83–14.29	98.3	2.49
Pheophytin b	20	1.82–13.58	96.7	3.05
Pheophytin b	12	2.98– 8.99	98.4	1.36
Lutein	12	0.50– 1.44	83.0	3.62
Carotene	20	0.80– 2.55	95.5	2.44
Solution B				
Chlorophylls a and b	12	3.91–13.99	94.7	2.10

Pheophytin a, pheophytin b, lutein, and carotene were prepared from spinach extracts, purified chromatographically, dissolved in petroleum ether, and used as stock solutions. A 10-ml portion containing varying proportions of the above pigments was chromatographed according to the proposed method. A control solution of each pigment was prepared by taking the same original quantity of each stock solution and making it up to the same volume with the same composition of solvent. A comparison between the eluate and the control solution gave a measure of the recovery of each pigment.

The recovery tests (Table 3) for chlorophyll were done separately, but in the same manner. The chlorophyll was eluted with acetone after the petroleum ether, 2% acetone in petroleum ether, and 3% acetone in petroleum ether had passed through the column. Since the eluted solution was in acetone, the stock solution that was in petroleum ether was evaporated under vacuum and redissolved in acetone.

The reproducibility of the method was determined by repeating the analysis twelve times on the same pigment extract (Table 4).

Table 4. Reproducibility of pigment analyses.

Pigment	No. of samples	Mean pigment content (mg/L)	Standard deviation	Coefficient variation (%)
Chlorophylls a and b	12	7.75	0.17	1.86
Pheophytin a	12	14.2	0.16	1.31
Pheophytin b	12	5.27	0.06	1.13
Lutein	12	1.32	0.02	1.56
Carotene	12	1.39	0.02	1.39

It is evident from Table 2 that pheophytins a and b, chlorophylls a and b, and carotene could be recovered with an accuracy of 95% or better over a relatively wide range of concentrations. The reproducibility tests (Table 4) showed much less variation than the recovery tests.

The recovery values for lutein were low compared to those for the other pigments. Further investigation revealed that repeated chromatography on lutein solutions always yielded another thin yellow band on the chromatogram. The second band had absorption maxima at 430, 454, and 482 mμ, compared with 430, 456, and 488 mμ for the main band. Possibly, this second band was an isomer of lutein that was formed continually from the main pigment. Further evidence of the change was obtained by analyses for lutein content at different time intervals (Table 5). After

Table 5. Relation between standing time and recovery of lutein.

Time at 40–44°F (hr)	Recovery (%)
4	94
12	85
16	83
72	78
120	71

five days at 40–44°F, only 71% of the lutein could be recovered although 98% of the pheophytin b could be recovered from the same solution. It is apparent that separation and determination had to follow pigment extraction without delay in order to achieve suitable accuracy. Some of the data on lutein in Table 2 were performed on extracts that had been allowed to stand overnight in the refrigerator. It was possible to obtain recoveries of 95% for lutein if the chromatographic separation followed the pigment extraction step without delay.

This method was developed primarily for estimation of chlorophylls a and b and pheophytins

a and b in order to follow color changes in processing. It is also useful for balance studies on the formation of pheophytins from the chlorophylls, since there may be some question about this conversion in processed spinach. It was also found that lutein and carotene could be estimated by the same procedure with very little extra work. Since these two pigments made up the major carotenoid portions and both contributed relatively little to the color changes, this phase of the method was not developed further.

RESULTS AND DISCUSSION

Table 6 shows the changes in color of spinach puree processed in a retort and an oil bath. There was no visual difference in the color of the samples processed at 240 and 250°F in a retort. This was borne out by the similarity in Delta E values. The samples processed in an oil bath did show a distinct color difference, and the Delta E values were progressively smaller as the temperature of processing was raised. The hue values indicated the same trend, and it was evident that the greatest change was in the "–a" attribute.

Table 6. Color measurements of thermally processed spinach puree.

Processing temperature (°F)	Color attribute				
	Adams coordinates[a]				Hunter coordinates[b]
	L	a	b	Delta E	Hue (–a/b)
Retort process					
Control	23.1	–10.8	20.4		1.05
240	21.8	– 0.6	17.2	10.8	0.14
250	22.0	– 1.0	17.4	10.3	0.15
Oil bath process					
Control	23.6	–11.4	20.0		0.923
240	21.9	– 0.2	17.7	11.6	0.087
250	21.7	– 3.5	16.8	8.7	0.366
260	21.5	– 5.7	17.8	6.4	0.534
270	21.9	– 8.2	18.2	4.0	0.657
280	22.2	– 8.2	17.8	4.1	0.682

[a] Data obtained with a Colormaster Differential Colorimeter and converted to the Adams Coordinate System.
[b] Data obtained with a Gardner Color and Color-Difference Meter.

Tables 7 and 8 show the changes in pigment content of spinach puree. In the retort process, the conversions of chlorophylls a and b to pheophytins a and b were comparable. There was some loss of lutein during processing, but very little loss of carotene. In the oil-bath process, the conversion of chlorophylls to their respective pheophytins was dependent on the temperature of processing. Chlorophyll a degraded to pheophytin a at a more rapid rate than chlorophyll b to pheophytin b (Table 9). Mackinney and Joslyn (1941) estimated that the rate of conversion of chlorophyll a was 7–9 times as fast as that of chlorophyll b in aqueous acetone extracts. Sweeney and Martin (1958) also found that chlorophyll a degraded more rapidly than chlorophyll b in experiments with various cooking times

Table 7. Pigment content (μg/g) of spinach puree processed in a conventional retort.

Pigment	Processing temperature			
	240°F		250°F	
	Control	Processed	Control	Processed
Carotene	58	58	53	47
Lutein	74	49	64	49
Pheophytin a	343	852	320	716
Pheophytin b	65	305	60	286
Chlorophyll a	519	39	400	36
Chlorophyll b	244	32	226	24

Table 8. Pigment content (μg/g fresh weight) of spinach puree processed in an oil bath.

Expt no.	Pigment	Processing temperature (°F)					
		Control	240	250	260	270	280
1	Carotene	50	48	45	45	46	46
	Lutein	57	47	51	54	54	57
	Pheophytin a	220	691	633	559	468	464
	Pheophytin b	65	236	173	142	111	118
	Chlorophyll a	426	39	105	167	236	236
	Chlorophyll b	204	51	107	136	167	168
	Total pheophytin a & b and chlorophyll a & b	915	1017	1018	1004	972	986
2	Carotene	53	45	54	53	51	51
	Lutein	64	47	55	58	57	59
	Pheophytin a	245	782	642	520	478	460
	Pheophytin b	62	254	157	146	115	108
	Chlorophyll a	455	36	143	198	243	263
	Chlorophyll b	191	34	115	125	149	156
	Total pheophytin a & b and chlorophyll a & b	953	1106	1039	989	985	987

Table 9. Ratio and percentage retention of chlorophyll b in spinach puree samples processed in a conventional retort and an oil bath.

Thermal process (°F)	Ratio Chlorophyll a / Chlorophyll b	Retention (%) of chlorophyll	
		Chlorophyll a	Chlorophyll b
Retort process			
Control	2.12	100	100
240	1.22	7.5	13.1
250	1.47	9.0	10.6
Oil bath process			
Control	2.23	100	100
240	0.92	8.0	20.2
250	1.12	26.8	53.6
260	1.40	40.6	64.8
270	1.52	53.7	78.5
280	1.55	55.5	80.3

Table 10. Color measurements of acetone-extracted, pigment-free spinach tissues.

Processing temperature (°F)	Color attribute			
	L[a]	a	b	Delta E
Retort process				
Control	57.01	0.6	13.1	
240	56.19	0.8	14.9	2.0
250	58.61	1.2	14.0	2.0
Oil bath process				
Control	58.26	1.8	12.9	
240	57.96	0.6	15.6	3.0
250	58.74	0.2	14.5	2.3
260	58.60	0.3	13.4	1.6
270	58.27	1.8	13.9	1.7
280	59.05	1.2	12.7	1.0

[a] Data obtained with a Colormaster Differential Colorimeter and converted to the Adams Coordinate System.

for frozen broccoli. In the present work, the difference in rate of conversion of the two chlorophylls was less than that given in the two previous citations. However, it is interesting that trends in chlorophyll conversion were similar in processes as different as cooking frozen broccoli and processing pureed spinach at a much higher temperature.

It is apparent in Tables 7 and 8 that appreciable amounts of chlorophyll had been degraded to pheophytin before the actual heat processing. This was unavoidable, because of the conditions of blanching, deaeration, and filling in this work, but this initial conversion could probably be minimized in actual practice.

The data in Table 8 indicate that lutein was also degraded to a greater extent at the lower temperatures. How much of the loss in pigment was due to a stereoisomerization and how much was due to actual degradation was not determined in this work, because no analytical method was developed for this isomer. In either case, the change in lutein would affect the color of the puree, though the color change would necessarily be very small. There was little or no change in the content of carotene at the different processing temperatures.

Table 10 shows the effect of processing temperature on the color of the pigment-free tissues. The tissues showed a greater color change between the processed and unprocessed (control) samples as the temperature of processing was lowered. This trend was evident only in the oil-bath samples, not in the retort samples. The effect of the processing temperature on the pigment-free tissues would affect the color of the puree, but, again, the color change would necessarily be very small.

Mackinney and Weast (1940) suggested that other degradation products of chlorophyll besides pheophytin might occur, because of the severity of the heat process in canned vegetables. Westcott *et al.* (1955) found small amounts of pheophorbide as well as pheophytin in commercially canned green beans, but Siegele (Gold and Weckel, 1958) found only pheophytin. This point could be checked in the present work by adding the total weight of pheophytins formed and chlorophylls degraded. It will be seen from Table 4 that the actual total weight of pheophytins and chlorophylls was somewhat higher in the processed samples than the total weight in the control samples. The analytical data indicated the presence of more pheophytin than could be accounted for by the degradation of chlorophyll. Since the discrepancy was greater at the lower temperatures, some substance was possibly being formed during processing that had higher absorption coefficients than the pheophytins and raised the apparent total pigment content. It would also have to be present as a contaminant of one of the zones on the

chromatogram. The error was not due to mere removal of magnesium from the molecule or to the type of error suggested by Van Norman (1957), because each would cause an error in the opposite direction. The cause of this discrepancy is not apparent at present.

It is evident from this work and that of others that foods containing chlorophyll can be packed with a high initial content of chlorophyll and an attractive color. It is equally true that the chlorophyll will be degraded to pheophytin relatively quickly upon storage. Chlorophyll-containing foods have been considered poor candidates for high-temperature short-time processing, because of the color retention problem and suggestions of flavor changes due to enzymatic activity (Adams and Yawger, 1961). It is hoped that a combination of conditions can be developed, including considerations of bacteriological sterilization, pigment stability, and enzymatic inactivation, such that the initial advantages of high-temperature short-time processing can be maintained.

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Protein-Bound Sodium and Potassium in Some Grains

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SUMMARY

Eight grain species were examined for sodium and potassium contents in protein and non-protein fractions before and after soaking. Most of sodium is protein bound, and potassium appears less strongly bound. Soaking does not seem to change these constituents in two fractions. Hardly any salt appears to leach out in soaking.

INTRODUCTION

In the past, sodium and potassium contents of food materials were determined only with the idea of acquiring a picture of mineral composition of foods (Clifford, 1955). Since the analytical procedure was tedious and time-consuming, a rough approximation sufficed. With the increase in low-sodium-diet therapy, food materials began to be examined carefully for sodium, and the literature on the subject became abundant (Roychowdhury and Gyani, 1959; Bills *et al.*, 1949; Food and Nutrition Board, 1954). In all these investigations, only the total amount of sodium and potassium present in foodstuffs has been reported; no one seems to have studied how these two elements are distributed in the protein and non-protein fractions of the grains, nor is there any indication as to how soaking and germination affect the distribution of the two minerals. It is quite apparent that organic constituents are prone to change chemically by germination (soaking), whereas such is not the case with inorganic constituents, though the redistribution of the inorganic constituents might be affected. With these objects, some grains were analyzed for sodium and potassium in protein and non-protein fractions, and also after soaking them for 48 and 72 hr.

EXPERIMENTAL

The grains were procured from the local market and washed superficially to remove adhering dust. Excess washing was avoided so leaching of salt from the body of the grains would be minimum.

The whole grain (with skin) was then immersed in an equal weight of sodium-free water in a flat basin, and left overnight. The excess water was then drained away and the moist grains were again stored for another 24 hr (room temperature, 20°C). One portion (2 g) was then dried 2 hr at 135°C for moisture determination (A.O.A.C., 1955), and the rest was left over for another 24 hr. After moisture determination, the grains were ashed at 500°C; sodium and potassium were determined by the method suggested previously (Roychowdhury and Gyani, 1959). The same was repeated with other lots (72-hr soaking) and in the unsoaked grains as well.

To precipitate proteins, the grains (both soaked and unsoaked) were ground with 10% trichloroacetic acid solution in a glass mortar. The treated mass was then centrifuged (3500 rpm) and the supernatant liquid was saved. The residue was treated in the same way 2–3 more times. The supernatant liquid portions (non-protein fractions) were combined in a basin and evaporated to dryness. The residue portion (protein fraction) was also dried. Both were ashed, and sodium and potassium were determined. The results are in Table 1.

DISCUSSION

Considerable sodium remained bound with proteins, although the trichloroacetic acid used in separating the proteins is quite a strong acid. One might have expected almost complete removal of amphoteric proteins, which are such weak acids.

Potassium appears to be less strongly bound with the proteins. There is some doubt whether the amounts that have been

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Table 1. Moisture content of the whole grains, and sodium and potassium in the protein and non-protein fractions. Results for sodium (mg/100 g) and potassium (g/100 g) are on dry powder basis, and moisture is in g/100 g. Botanical names are given in parentheses.

Grains	Hours of soaking	Moisture	Sodium		Potassium	
			Non-protein fraction	Protein fraction	Non-protein fraction	Protein fraction
Barley *(Hordeum vulgare)*	0	11.0	8.7	4.9	0.22	0.02
	48	39.1	7.0	4.7	0.21	0.02
	72	38.2	6.5	4.2	0.20	0.02
Wheat, whole *(Triticum aestivum)*	0	10.5	5.5	2.6	0.22	0.02
	48	36.5	5.0	2.5	0.21	0.02
	72	36.4	4.7	2.5	0.20	0.02
Bengal gram *(Cicer arietinum)*	0	9.7	24.6	3.8	0.55	0.06
	48	53.9	23.6	3.3	0.54	0.06
	72	52.2	21.8	3.2	0.54	0.06
Black gram (Urd) *(Phaseolous mungo)*	0	10.0	13.3	8.5	0.65	0.15
	48	51.0	12.7	6.5	0.57	0.16
	72	49.5	12.2	6.0	0.55	0.16
Green gram (Mung) *(Phaseolous aureus* Roxb.*)*	0	10.5	7.5	12.2	0.86	0.10
	48	60.8	5.7	4.8	0.85	0.08
	72	59.2	5.7	4.8	0.85	0.08
Lentil (Masur) *(Lens culinaris* Medic.*)*	0	10.5	5.5	4.5	0.45	0.04
	48	51.4	5.0	3.5	0.41	0.03
	72	50.9	4.8	3.5	0.40	0.03
Lathyrus (Khesari) *(Lathyrus sativus)*	0	7.3	23.2	7.7	0.68	0.05
	48	55.6	19.8	7.5	0.64	0.04
	72	55.6	19.2	7.0	0.62	0.04
Peas (Matar) *(Pisum sativum)*	0	9.9	10.8	6.8	0.60	0.10
	48	54.9	10.0	4.5	0.58	0.09
	72	54.3	9.2	4.0	0.57	0.09

determined in protein and non-protein fractions represent the correct quantity or the redistribution that prevails after disturbance by the presence of trichloroacetic acid. If a disturbance is produced, it is possible that more potassium is being freed from proteins than sodium. The reason for the same may be found in a physical effect like diffusion, since, although K ions have a larger radius (1.33Å) than Na ions (0.95Å) in a crystal, the sodium ions become larger through hydration.

It also appears that hardly any salt is leached out by soaking, for though the sum of the Na and K after soaking does not equal the sum before soaking, the change is very slight and consistent. The sodium figures for Mung (green gram) and peas are exceptions.

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Inositol and Free Sugars in Chicken Muscle Post-Mortem

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SUMMARY

Chicken muscle was analyzed for free sugars using separations by paper chromatography. Inositol, glucose, sedoheptulose, mannose, fructose, ribose, and ribulose were identified. Two other unknown compounds appeared to be arabinose and xylose. Five minor components detected were not identified.

The principal free sugars present in chicken muscle immediately post-mortem were glucose and fructose. Ribose occurred only in trace amounts. During a 6-day storage period at ice temperature, glucose levels increased in white muscle from 13-week-old pullets and also in both red and white muscle from old hens, but decreased in red muscle from pullets. Red muscle contained more than twice as much free inositol as white muscle. Inositol, fructose, and ribose increased during storage in the two kinds of muscle in both older and younger birds.

There is increasing evidence that sugars and sugar derivatives are precursors of some of the substances contributing to the normal cooked flavor of meats (Batzer *et al.*, 1960; Wood, 1961). Solutions containing reducing sugars and amino acids develop meat-broth-like flavors on heating (Lobanow and Wolfson, 1958; May, 1960; Morton *et al.*, 1960; Wood, 1961). Reducing sugars, however, also participate in reactions causing deteriorative changes in meat and fish products (Hendrickson *et al.*, 1956; Jones, 1958; Sharp, 1957; Tarr, 1954).

The purpose of the present study was to determine the individual sugars present in chicken meat, and to determine the variations that may occur in the principal free sugars and inositol with respect to type of muscle, age of bird and time post-mortem.

EXPERIMENTAL

Samples. Extracts for the qualitative analysis of free sugars were prepared from fresh and frozen muscle samples of Single Comb White Leghorn and New Hampshire chickens of both sexes and various age groups. To study quantitative occurrence of inositol and free sugars post-mortem, two 13-week-old New Hampshire pullets, and two 18-month-old New Hampshire hens, were used for the analyses. Each bird was slaughtered by severing the jugular vein and bleeding for two minutes. The red muscles of the left leg and thigh were immediately cut out, weighed, sliced, and homogenized. The time required from bleeding to completion of homogenization was 15 min. The white muscles of the left breast were then excised and treated in the same manner. Homogenization of these muscles was completed in 30 min post-mortem. The remainder of the carcass was then eviscerated and packaged in a polyethylene bag to prevent leaching of the tissue constituents, and was covered with crushed ice in a Dewar flask. For comparison, muscles from the right side were removed from 2 of the birds after 24 hr at 1°C, and from 2 others after 6 days at this temperature. Muscle from the right half was compared with muscle from the left half of the bird in each instance.

Extraction. Muscle was homogenized in a Servall Omni-Mixer with small amounts of added water to give a homogenate of creamy consistency. Picric acid was added directly to muscle that was to be analyzed quantitatively. In either instance, four parts of 1% picric acid solution was added to one part of muscle. The mixture was centrifuged, filtered, and extracted with purified petroleum ether to remove lipid material.

Deionization. Each extract was passed over a column of Dowex 50(H), 200–400-mesh. The effluent and washings were treated with Amberlite 1R 45(OH), 200–400-mesh. The solution was stirred with 3/5 of the latter resin, and then passed over the remainder of the resin in column form. In freshly excised muscle, glycogen was present in sufficient amounts to interfere with

Table 1. Substances detected by sugar reagents in extract[a, b] from frozen chicken breast.

Sugar	Distance from starting line	Detecting reagents	Color	Remarks
unknown cmpd.	0.6 cm[c]	$AgNO_3$[d], A-P[e]	brown	Trace
unknown cmpd.	2.0 cm	$AgNO_3$, A-P	brown	Trace
unknown cmpd.	4.5 cm	$AgNO_3$, A-P	brown	Trace
inositol	6.6 cm	$AgNO_3$, N & K[f]	brown red-orange	Major constituent Specific for inositol
unknown cmpd.	8.5 cm	$AgNO_3$, A-P orcinol[g]	brown tan	Trace
glucose	16.1 cm	$AgNO_3$, A-P A-H-P[h]	brown tan	Major constituent
sedoheptulose	19.0 cm	orcinol	blue	Trace
mannose	20.4 cm	$AgNO_3$, A-P A-H-P	brown brown	Minor constituent Color develops slowly
fructose	22.2 cm	$AgNO_3$, A-P orcinol resorcinol[i]	brown tan red-brown	Major constituent
unknown cmpd.	24.6 cm	$AgNO_3$ A-P	brown red	Trace Possibly arabinose
unknown cmpd.	28.0 cm	$AgNO_3$, A-P	brown	Trace, possibly xylose
ribose	32.7 cm	$AgNO_3$, A-P A-H-P	brown red	Major constituent
ribulose	36.0 cm	$AgNO_3$, A-P resorcinol orcinol, A-D-A[j]	brown bluish-gray tan	Minor constituent
unknown cmpd.	46.0 cm	$AgNO_3$	brown	Trace

[a] Muscle from year-old Single Comb White Leghorn hen stored at 5°F.

[b] Solvent system was ethyl acetate-acetic acid-water (3:1:1 v/v).

[c] Distances are listed instead of Rg values because descending-type chromatograms do not give reproducible Rg values.

[d] Smith, 1958.

[e] Aniline-phosphoric acid (Bryson and Mitchell, 1951).

[f] Nagai and Kimura reagent (1958).

[g] Bevenue and Williams, 1951.

[h] Aniline hydrogen phthalate (Partridge, 1949).

[i] Bryson and Mitchell, 1951.

[j] Aniline diphenyl amine (Smith, 1958).

chromatography. It was removed from the deionized extracts by evaporation to dryness and precipitation from 2:1 ethanol-water. Extracts were concentrated so that 1 ml was approximately equivalent to 2 g of moisture-free muscle.

Chromatography. Descending chromatograms were run on Whatman No. 1 paper. The solvent systems used were: 1) ethyl acetate–acetic acid–water (3:1:1 v/v); 2) butanol-acetic acid–water (60:15:25 v/v); 3) ethyl acetate–pyridine–water (6:1:1 v/v). For quantitative analyses, solvent systems 1 and 2 were used. Appropriate detection reagents were used to locate standards, and paper sections were cut out in the area corresponding to the known standard and eluted with water. This procedure was employed for the quantitative estimation of glucose, inositol, and ribose.

Analytical. Glucose and inositol were determined by the periodate oxidation method of Hirst and Jones (1949). Acetic acid was removed from the filter paper by placing it under reduced pressure in the presence of moisture (Laidlaw and Reid, 1952). Losses of formic acid produced during the digestion were prevented by using ground-glass tapered flasks fitted with cold fingers.

Inositol was also estimated by the method of Dixon and Lipkin (1954) as modified by Agranoff *et al.* (1958).

Ribose was determined by the orcinol method of Mejbaum (1939). Filter paper contains soluble orcinol-reacting substances that are difficult to remove by preliminary washing of the paper (Dubois *et al.*, 1956). The ribose determinations are therefore only reasonably approximate (10% error) in those cases where the value obtained was 5 mg/100 g dry muscle or greater.

Keto sugars (principally fructose) were determined by the method of Roe (1934).

RESULTS AND DISCUSSION

Free sugars in chicken muscle. The following substances were found consistently in fresh and frozen red and white muscle samples from birds of several ages and both sexes: inositol, glucose, fructose, ribose, and mannose. Sedoheptulose, ribulose, two substances that appeared to be xylose and arabinose, and several unknown compounds were found in each type of sample, but did not appear consistently in all samples.

Table 1 lists substances found in the water extract of breast muscle of a 1-year-old White Leghorn hen that had been stored in the frozen state for 18 months. This sample contained all the compounds mentioned above.

Post-mortem changes. Values for free inositol, glucose, fructose, and ribose are shown in Table 2 for muscle of birds just after killing and for muscle of the same birds after storing at 1°C for 1 and 6 days.

Inositol levels were somewhat higher in muscle of younger birds than in comparable muscle of older birds. Red muscle contained more than twice as much inositol as white muscle. In all samples, free inositol increased during storage. Levels of free inositol in muscle tissue appear to be dependent upon the age of the animal, the type of muscle, and the time post-mortem. Most of the data in the literature for inositol in muscle of various species do not consider these variables, although Needham (1923, 1924) noted that free inositol increased post-mortem in rabbit and rat muscle.

Glucose was the principal free sugar in fresh-killed and chill-stored chicken meat (Table 2). In breast muscle of both older and younger birds and in dark muscle of older birds, glucose increased during cold storage and approximately doubled during 6 days at 1°C. A decrease in the glucose content of the leg and thigh muscle of the younger birds during storage was unusual in that it did not follow the pattern of the other samples.

Since fructose is the only important keto sugar in chicken muscle (Table 1), the results in Table 2 indicate that fructose increased in all samples during storage.

Ribose was present only in trace amounts in muscle of freshly killed chicken, and, although it increased during storage, it was still present at very low levels (Table 2). In this respect chicken differs from beef, veal, and fish. In certain fish free ribose is found only post-mortem, and may increase to levels higher than those of glucose (Tarr, 1954; Jones, 1958). Ribose content of beef and veal, though variable, appears to be much higher than that of chicken (Tarr, 1954; Grau *et al.*, 1960). According to Wood (1961), ribose is an important flavor precursor in beef. Considering the low levels of ribose in chicken

Table 2. Inositol and free sugars of chicken muscle—effect of storage at 1°C for 1 to 6 days.

	Leg and thigh				Breast			
	Young [a]		Old [b]		Young		Old	
Mg inositol/100 g dry muscle								
Fresh [c]	122[d]	126[e]	86[d]	87[e]	52[d]	48[e]	39[d]	33[e]
24 hour storage at 1°C	148	161	120	120	72	72	50	49
Fresh	135	140	117	120	54	57	26	28
6 day storage at 1°C	188	201	147	149	80	82	38	41
Mg glucose/100 g dry muscle [e]								
Fresh	188		155		141		105	
24 hour storage at 1°C	139		186		236		272	
Fresh	156		122		118		115	
6 day storage at 1°C	116		298		289		272	
Mg keto sugar/100 g dry muscle as fructose [f]								
Fresh	10		15		13		13	
24 hour storage at 1°C	22		24		44		42	
Fresh	15		8		13		9	
6 day storage at 1°C	22		39		50		40	
Mg ribose/100 g dry muscle [g]								
Fresh	1		1		3		less than 1	
24 hour storage at 1°C	4		3		5		1	
Fresh	1		1		1		1	
6 day storage at 1°C	9		6		14		7	

[a] Thirteen-week New Hampshire pullets.
[b] Eighteen-month-old New Hampshire hens.
[c] Leg and thigh, 15 min post-mortem; breast, 30 min post-mortem.
[d] Method of Dixon and Lipkin (1954) as modified by Agranoff *et al.* (1958).
[e] Method of Hirst and Jones (1949).
[f] Method of Roe (1934).
[g] Method of Mejbaum (1939).

Table 3. Periodate oxidizable substances in glucose equivalents (mg/100 g dry muscle).

	Leg and thigh						Breast					
	Young [a]			Old [b]			Young			Old		
	Obs.[c]	Calc.[d]	Diff.[e]	Obs.	Calc.	Diff.	Obs.	Calc.	Diff.	Obs.	Calc.	Diff.
Fresh [f]	328	309	19	268	244	24	214	195	19	153	143	10
24-hour storage at 1°C	301	301	0	302	211	9	335	332	3	358	342	16
Fresh	312	292	20	242	236	6	190	179	11	161	147	14
6 day storage at 1°C	329	317	12	484	462	22	421	407	14	362	346	16

[a] Thirteen-week-old N.H.
[b] Eighteen-month-old N.H.
[c] Periodate-oxidizable substances in glucose equivalents by method of Hirst and Jones (1949).
[d] Total reducing substances by sum of inositol, glucose, keto sugar, and ribose as determined individually but recalculated in terms of glucose equivalents by periodate oxidation method.
[e] Difference between observed and calculated.
[f] Leg and thigh, 15 min post-mortem; breast, 30 min post-mortem.

it would seem that glucose and fructose may be more important than ribose as flavor precursors in chicken.

The post-mortem changes in free sugars of chicken muscle are similar to those reported for fish (Tarr, 1954; Jones, 1958; Burt, 1961) and veal (Grau *et al.*, 1960).

Estimation of total reducing sugars. Samples of the various extracts were analyzed for total reducing substances by the periodate method. The results, in glucose equivalents, were compared with the sum of the components determined individually, which were also expressed as glucose equivalents. These data (Table 3) indicate that all but minor amounts of reducing substances were accounted for by inositol, glucose, fructose, and ribose. If a specific correction for inositol is applied, this method would appear to give a more reasonable estimate of free reducing sugars in muscle than the usual nonspecific methods for reducing sugars.

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Enzymatic Degradation of Collagen[a]

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SUMMARY

Ficin cannot hydrolyze native collagen. Collagen can be degraded by this enzyme only after it has been denatured by heat, low pH, or high salt concentrations. This appears to be true also for the action of bromelain, papain, and trypsin on collagen.

The use of proteolytic enzymes for tenderizing meat is increasing rapidly. This tenderizing effect may depend to some extent on the ability of the enzymes to degrade collagen. It is not clear from the literature, however, whether proteolytic enzymes other than the *Clostridium* collagenases can degrade collagen.

Investigations of a series of bacterial and mold enzymes by Mandl *et al.* (1958) indicated that, under physiological conditions of pH and temperature, only the enzymes produced by *Clostridium histolyticum* and *Clostridium welchii* are capable of hydrolyzing native collagen. This is supported by data of Dresner and Schubert (1955), Neuman and Tytell (1950), and Sherry *et al.* (1954). Springell (1955), however, reported that native collagen is comparatively resistant to both *Clostridium histolyticum* collagenase and trypsin.

Collagen appears to be readily digested by pepsin in acid solution (pH 1-2) (Sizer, 1949a; Neuman and Tytell, 1950). Pepsin at pH 1.5–1.7 has been used to determine the quality of meat. Smorodintsev (1934) and Smorodintsev *et al.* (1939) found that first-grade meat is digested 20–30% better than second-grade meats, because of an approximately 65% decrease in rate of hydrolysis of collagen fibers compared with pure muscle fibers (Smorodintsev and Adova, 1935). In general, the results of Grau and Hamm (1951) support this conclusion. Observed pepsin action on collagen may be due to an attack on denatured collagen formed by the acid conditions used in the assay (Gustavson, 1956; Fokina, 1937; Cassel and McKenna, 1954; Courts, 1960).

Collagen is found to be relatively resistant to hydrolysis by trypsin (Grassman *et al.*, 1937; Neuman and Tytell, 1950; Sizer, 1949b; Springell, 1955; Mandl *et al.*, 1958; Dresner and Schubert, 1955; Gustavson, 1956) and by chymotrypsin and papain (Neuman and Tytell, 1950).

On the other hand, Wang *et al.* (1958), by use of histological techniques, found that ficin, papain, bromelin, and trypsin, when used at high concentrations (2.5–5.0%), possess collagenase activity at 23–25°C. The microbial enzymes HT proteolytic, Hydralase D, and Hydralase TP had little or no collagenase activity. Miyada and Tappel (1956b) reported that bromelin, ficin, trypsin, Rhozyme P-11, and papain all digest collagen of freeze-dried biceps femoris muscle when incubated 1 hr at 60°C. Sherry *et al.* (1954) found that papain and ficin digested collagen at pH 2.0–4.5. However, they presented evidence that the native collagen at the low pH undergoes a reversible alteration that renders it susceptible to these enzymes. Grant and Alburn (1960) reported that trypsin, chymotrypsin, elastase, and endopeptidase from procarboxypeptidase A all solubilized collagen in the presence of calcium salts, salicylates, arginine, creatinine, and guanidine.

This work was conducted to provide detailed information on the collagenase activity of ficin and to compare this activity with

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the collagenase activity of bromelain, papain, trypsin, HT proteolytic, and fungal protease.

MATERIALS AND METHODS

Collagen was prepared by method B of Baker *et al.* (1954) from Achilles tendons of cattle. The final purified product was freeze-dried for 3 days in a Stokes 2004L lyophilizer at a pressure of 0.1 mm and plate temperature of 27°C. It was then mixed with a large amount of dry ice and passed through a Wiley Mill equipped with a 20-mesh screen. The collagen was stored at −27°C. Kjeldahl nitrogen content was 17.1%, which is in agreement with reported values (Miyada and Tappel, 1956a, 16.98%; Salo, 1949a, 17.25%; Jacobson and Lollar, 1951, 17.2%). Moisture content was 6.0% as determined in a vacuum oven.

Ficin was obtained from Merck and Co., HT proteolytic and fungal protease enzymes from Miles Chemical Co., trypsin (2X crystalline) from Nutritional Biochemicals Corporation, papain ("Panol," purified) from Biddle Sawyer Corporation, and bromelain from Dole Corporation.

Measurement of activity was very similar to the method used by Mandl *et al.* (1958). Collagen, ranging from 25 to 150 mg, was weighed into 13 × 100-mm tubes. Then the following were added to each tube: 0.10 ml versene (0.25*M*), 0.10 ml mercaptoethanol or cysteine (0.25*M*), 0.40 ml buffer (0.5*M*), other additives, enzyme, and water to bring the total to two ml. For nonsulfhydryl enzymes, versene and mercaptoethanol were replaced with water. After a reaction period of 30 min with stirring, 3 ml of 5% trichloroacetic acid were used to stop the reaction. The tubes were allowed to stand for one hour and then centrifuged, and the supernatant liquid removed. Amount of proteolysis was determined on the supernatant liquid by optical density measurements at 280 mμ, ninhydrin method (Moore and Stein, 1954), and in certain cases by the micro-Kjeldahl method as modified by Johnson (1941). Reported values are averages of 2–5 experiments. pH was determined on separate reaction mixtures with a Beckman Model G pH meter.

RESULTS AND DISCUSSION

Effect of pH and temperature. Effect of pH and temperature on solubilization of collagen by ficin is shown in Tables 1 and 2 and Figs. 1 and 2. Only a small amount of collagen is solubilized by ficin at pH 4.5 and above. The percentage solubilized at pH 7.0 and 35°C is independent of ficin concentration (0.5–2.0 mg) (Fig. 2) and the amount of collagen used (25–150 mg collagen per assay tube), indicating that a small fraction (approx 10%) of the collagen is capable of being solubilized under these conditions but that the remainder cannot be attacked. Trypsin, bromelain, and papain were also able to solubilize collagen to the same extent under these conditions (Fig. 1). Below pH 4.5, collagen is solubilized quite rapidly, with a pH optimum near 2.9 at 35°C and an incubation time of 30 min. Two mg ficin is able to solubilize 25 mg collagen completely in 30 min at 35°C and pH 2.9 (Fig. 2). Study of the split products formed under the conditions just described indicates that only 4.3% of the peptide linkages involving α-amino acids are split (ninhydrin assay at 570 mμ).

If a sample of collagen is held 1 hour at pH 2.03 and 35°C and then treated with ficin after the pH is adjusted to 6.48, there is 49% solubilization of collagen, compared to 8.75% with a sample held for 1 hour at pH 6.48 and 35°C before enzyme treatment (Table 2). The available evidence indicates that ficin is able to digest collagen at pH 2–4.5 because there is a slow denaturation of collagen in this pH range. This

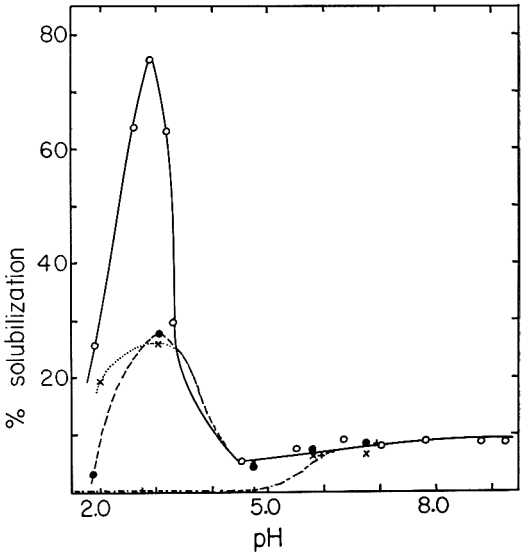

Fig. 1. Effect of pH on solubilization of collagen by ficin (○), bromelain (●), papain (X), and trypsin (+). Reactions carried out at 35°C for 30 min, 25.0 mg collagen, 0.50 mg enzyme, and reaction volume of two ml. $1.25 \times 10^{-2}M$ versene and mercaptoethanol as activators for ficin, bromelain, and papain.

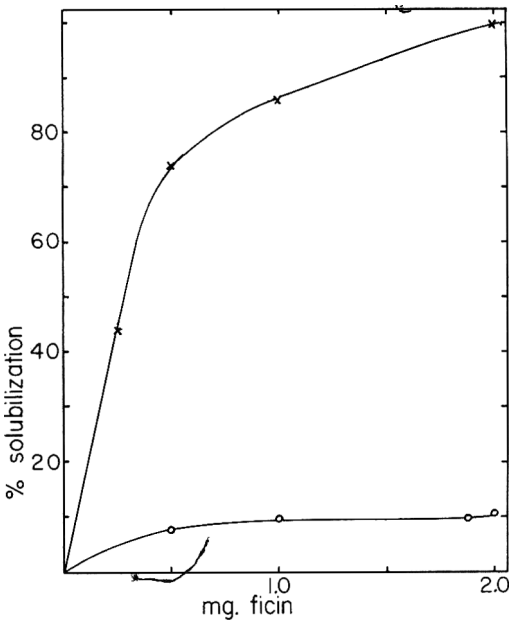

Fig. 2. Effect of ficin concentration on percent solubilization of collagen at pH 7.0 (○), and pH 2.86 (X). Reactions carried out at 35°C, for 30 min, 25.0 mg collagen, and reaction volume of two ml. $1.25 \times 10^{-2} M$ versene and mercaptoethanol as activators.

is in agreement with results of Sherry *et al.* (1954). The degree of solubilization falls off on the acid side (below pH 2.9) because of rapid inactivation of the enzyme, not because the collagen is not denatured. This is indicated by the effect of temperature on the amount of solubilization by ficin (Table 1).

While the temperature optimum for solubilization of collagen by ficin is near 35°C at pH 2.9, at higher pH values, where ficin is more stable, there is a marked increase in percentage solubilization at 60°C (Table 1). That this increase in solubilization is due to denaturation of collagen with subsequent enzymatic activity can be shown by holding a suspension of collagen (pH 5.7) for 30 min at 60°C, and then treating with ficin for 30 min at 35°C. There is a 35% solubilization of collagen under these conditions, the same as when the enzymatic solubilization is carried out at 60°C.

Effect of other enzymes. Bromelain and papain also solubilize collagen maximally at pH 2–4.5. Trypsin shows no activity in this range. At pH 6–7 all these enzymes show the same slight solubilization of collagen. HT proteolytic and fungal protease enzymes showed no activity on collagen even though they degraded casein quite rapidly.

Effect of other factors. Gross *et al.* (1955) showed that the extent of digestion of collagen is dependent on the method of preparation. Data of Table 2 indicate that the methods of preparation used in the present work did not influence the degree of solubilization.

Increase in sodium chloride concentration produced a small but significant increase in solubilization of collagen (Table 2). This is in agreement with the results of Hamm (1955) and Sizer (1949b). The effect of sodium chloride is to increase the solubility of collagen (Pavlov, 1938; Salo, 1949b; Harrington, 1958). Calcium chloride did not increase the solubilization of collagen by ficin (Table 2); in fact, there was a small decrease. This is in contrast to the report of Grant and Alburn (1960) that calcium chloride increased not only the solubility of rat-tail collagen but its digestibility by trypsin, chymotrypsin, elastase, and endopeptidase from procarboxypeptidase A. Lysine did not affect the degree of solubilization of collagen by ficin, but there was a significant increase in the presence of arginine (Table 2). The latter is in agreement with the results of Grant and Alburn (1960).

Ficin, bromelain, papain, and trypsin cannot solubilize collagen until it has been denatured. Denaturation produces a swelling of the collagen fibers so that enzymes can penetrate and attack them. Collagen in meat is not attacked by these enzymes at low temperatures, but is degraded quite rapidly at elevated temperatures (60–70°C). If the meat is properly cooked, the collagen will also be converted to gelatin under the influence of moist heat and will not contribute to toughness. On the other hand, elastin becomes more rigid on heating. It has recently been shown that elastin is solubilized quite rapidly by ficin even at low temperatures (Yatco-Manzo and Whitaker, 1962). Conditions for the solubilization of elastin by ficin are optimum at pH 5.5 and 55°C.

Table 1. Effect of pH and temperature on solubilization of collagen with ficin.[a]

Temp. (°C)	% Solubilization at pH:								
	1.90	2.60	2.86	3.30	4.50	5.50	6.95	7.80	9.20
25			43.8						
35	25.8	63.7	75.5	29.5	5.44	7.31	7.78	8.90	8.44
40			62.0	24.4	7.50	8.90	10.8	11.1	10.7
45			42.3	33.3	16.2	15.0	12.7	12.7	13.1
50			35.1						
55			25.2						
60			0.94	28.5	36.8	35.4	33.3	40.4	35.4

[a] Reactions carried out for 30 min, 25.0 mg collagen, 0.50 mg enzyme, and reaction volume of two ml. $1.25 \times 10^{-2}M$ versene and mercaptoethanol as activators.

Table 2. Effect of several factors on solubilization of collagen by ficin.

Factor	Condition	% solubilization
Method of preparation of substrate [a]	Freeze-dried tendon [b]	9.00
	Ground freeze-dried tendon [c]	11.6
	Purified native collagen [d]	8.62
Prior pH treatment [e]	1 hr at pH 6.48, 35°C	8.75
	1 hr at pH 2.03, 35°C	49.1
Additive	NaCl (M) [f]	
	0.0	3.20
	0.2	5.95
	0.4	7.02
	0.8	9.24
	$CaCl_2$ (M) [g]	
	0.0	16.4
	0.16	13.3
	0.32	12.8
	0.40	11.2
	0.2M lysine [g]	15.7
	0.2M lysine + 0.1M $CaCl_2$ [g]	14.4
	0.2M arginine [g]	33.1
	0.2M arginine + 0.1M $CaCl_2$ [g]	33.2

[a] Reaction carried out at pH 8.0, 35°C, for 30 min, 25.0 mg collagen, 1.0 mg enzyme, and reaction volume of two ml. $1.25 \times 10^{-2}M$ versene and cysteine as activators.

[b] Tendons cut into approx 2×2-mm pieces, ether-extracted, and freeze-dried.

[c] As in b except ground through Wiley Mill using 20-mesh screen.

[d] Prepared by method B of Baker *et al.* (1954).

[e] After pH treatment, reaction carried out at pH 6.48, 35°C, for 30 min, 25.0 mg collagen, 0.50 mg enzyme, and reaction volume of two ml. $1.25 \times 10^{-2}M$ versene and mercaptoethanol as activators.

[f] Reaction carried out at pH 7.0, 35°C, for 30 min, 150.0 mg collagen, 0.50 mg enzyme, and reaction volume of two ml. $1.25 \times 10^{-2}M$ versene and mercaptoethanol as activators.

[g] Series of reactions carried out with different preparation of ficin. Reaction mixture contained 25.0 mg collagen, $1.25 \times 10^{-2}M$ versene, and cysteine and additive at pH 5.7. Incubated 1 hour at 25°C. Reaction with ficin carried out at 35°C, for 30 min, 0.50 mg enzyme, and reaction volume of two ml.

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The Molecular Oxygen Content of Dehydrated Foods [a]

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SUMMARY

A polarographic method for measuring the molecular oxygen content of foods is described. Oxygen contents determined by the method were of the same order of magnitude as peroxide oxygen values of non-rancid fats and oils. Factors that controlled the molecular oxygen content of dehydrated foods were percent moisture, surface area, partial pressure of oxygen, and absorbability of other gases in the headspace.

One reason for the interest of the U. S. armed forces in dehydrated foods, aside from the logistic advantage, is the desire to have rations with long storage life. Oxygen limits the stability of dehydrated foods by initiating reactions that lead to such defects as rancidity, loss of nutritional value, and discoloration. We therefore investigated methods for measuring molecular oxygen in dehydrated foods, and the conditions that affect their affinity for oxygen.

It is common practice to measure the uptake of oxygen by foods at elevated temperatures with the Warburg apparatus or by similar techniques. Frequently, this is done without regard to the fate of the oxygen after uptake. The purpose of this work was to measure the oxygen that is held by the food and remains in the molecular form still unreacted with the food components. This oxygen may be held by physical entrapment, by chemisorption, or, to a lesser degree, by physical adsorption. It seems reasonable that molecular oxygen in close proximity to oxidizable components of the foods may be responsible for initiating oxidative deterioration. Knowledge of this oxygen content might therefore provide advance information on the inherent stability of a product.

Considerable attention has been given to direct measurement of the oxygen content of biological materials (Brezina and Zuman, 1958; Kolthoff and Lingane, 1952) but not of dehydrated foods. Usually, the molecular oxygen content of dehydrated foods is estimated indirectly by measuring the concentration of oxygen released to the headspace. During the release of oxygen the food product may be enclosed in an evacuated chamber (Haller and Holm, 1947) or may be placed in an atmosphere of nitrogen (Lea *et al.*, 1943). The Winkler manganous hydroxide method (APHA, 1955) was used for some of the work reported here. Because of its limitations in this application, however, most of the work was done by the polarographic method described below. Since the polarographic method was responsive to differences in moisture content, processing, and headspace gas composition, it offers promise of being useful in stability studies and in defining characteristics that would be expected to provide good stability.

EXPERIMENTAL

The standard Winkler method for dissolved oxygen (APHA, 1955) was found to be appli-

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cable to measurement of the oxygen content of starch dispersed in deoxygenated water. In this method, dissolved oxygen converts manganous ion to manganic ion, which in turn releases iodine from potassium iodide in amounts equivalent to the dissolved oxygen. The iodine is measured by titration with standard sodium thiosulfate solution. The starch served as the indicator. This method was not applicable to dehydrated foods.

Applicable to several materials was a modification of the polarographic method described by Petering and Daniels (1938) and by Karsten (1939) for measuring the oxygen content of soils. In principle, this method depends on the reduction of molecular oxygen at the dropping mercury electrode. In our modification, the dropping mercury electrode was inserted approximately 1½ in. through a rubber stopper into a 1-oz jar containing 30 ml of deoxygenated 0.1*N* potassium chloride solution and a Teflon-covered 1-in. magnetic stirring bar. The solution, contained initially in an 8-L Pyrex glass reservoir, was deoxygenated by sparging with water-pumped nitrogen from which traces of oxygen were removed by passing over hot copper turnings. The 1-oz jar was filled by siphoning from the reservoir. The other half of the cell consisted of a Hildebrand-type saturated calomel electrode, the arm of which was inserted through the rubber stopper.

After measurement of the residual current flow (see "Circuitry," below), a 1.00-g quantity of pulverized food was introduced into the jar. The stirring bar was then actuated at intervals by moving a second bar below the jar in such a manner that the surface of the food-electrolyte suspension was not agitated. The seal provided by the rubber stopper was depended upon to maintain an air-tight system during equilibration of the oxygen of the food solids with the deoxygenated solution. Three current readings, taken between stirring intervals, were recorded and averaged. Equilibration was considered to be complete when the range of readings agreed within one galvanometer scale division, equivalent to 0.047μl of oxygen per gram. Readings of $\pm$0.05μl of oxygen per gram represent the maximum reproducibility of the method, in its present state, for duplicate samples. The difference in diffusion current, measured before and after addition of the sample, was related to the oxygen content by a calibration curve. All measurements were made at room temperature.

Electrodes. In addition to the dropping mercury electrode, which has been used extensively in measurement of the dissolved oxygen content of biological media (Brezina and Zuman, 1958; Kolthoff and Lingane, 1952), other measurements were made with a self-contained platinum cathode-silver anode electrode system covered with a polyethylene membrane (Watanabe and Leonard, 1957). Use of this electrode permitted continuous rotation of the bar magnet during current measurements. Equilibrium current values as shown by stable readings during a 6-min interval were recorded.

Circuitry. Earlier measurements with the dropping mercury electrode were made with a simplified electrical circuit similar to that described by Heyrovsky (1951). Later measurements with either the mercury or platinum electrode were made with a recording Model XXI Sargent polarograph (no endorsement implied).

Specificity of the method for molecular oxygen. Some specificity was assured by making all measurements at an applied potential of —0.6 volt, which is on the plateau of the first of the two waves for oxygen given by the dropping mercury electrode (Kolthoff and Lingane, 1952). At this potential, molecular oxygen is reduced to hydrogen peroxide. In the case of the platinum electrode there is only one oxygen wave, and the only reaction occurring in pure oxygenated solutions is reduction of oxygen to hydrogen peroxide (Brezina and Zuman, 1958; Kolthoff and Lingane, 1952).

The specificity of the method was confirmed by the addition of glucose oxidase and glucose to food-electrolyte suspensions containing appreciable quantities of oxygen as shown by current measurements. In all cases tested, a second measurement of the current showed negligible oxygen values with either electrode.

Calibration. Calibration was against 0.1*N* potassium chloride solutions containing a range of dissolved oxygen concentrations. The reference method was the standard Winkler method (APHA, 1955).

Desiccation of samples. One-gram samples of the product were conditioned for approximately one week at 23% relative humidity at 22°C. The samples were then transferred to 0%-relative-humidity desiccators charged with magnesium perchlorate. Samples were withdrawn at intervals for oxygen determinations. Moisture contents were calculated from the initial moisture values by following the weight changes in the desiccator. All products were conditioned at 72°F to avoid a difference between the temperature of conditioning and the temperature of the oxygen measurements.

Moisture-sorption isotherms. The desiccator-equilibrium method described by Slawson and Salwin (1958) was used. Relative-humidity values corresponding to the moisture contents were determined from the isotherms.

Moisture determinations. Starch samples were

dried overnight at 70°C in a vacuum oven. Meat samples were either dried by this procedure or for 5 hr at 100°C in a vacuum oven.

Monomolecular moisture-layer values. These values were calculated by the method described by Salwin (1959). The corresponding relative-humidity value was then determined by inspection of the isotherm.

Bulk volume. Six grams of the material were transferred to a graduated cylinder, which was then shaken and tapped in such a manner as to settle the contents but not to pack them. The bulk volume was recorded as cc per gram.

MATERIALS

Starch. This material was reagent-grade soluble potato starch containing 7.0% moisture.

Beef, freeze-dried. Three 95-g portions of a thoroughly mixed ground beef sample (Cutter and Canner Grade) were transferred to three 600-ml beakers. Two portions were preheated to internal temperatures of 60°C and 98°C; the third was left in the uncooked state. Each portion was then blended for 1½ min with 190 ml of distilled water, shell-frozen in a round-bottomed flask, and dehydrated in a laboratory lyophilizer. The dried material was then reground in a sharp-bladed food blender. The resulting product averaged 3.5% moisture. The fat content of all samples, regardless of preheat treatment, was 20.2%.

Chicken, freeze-dried. The entire muscular tissue, skin excluded, of an all-purpose roaster or frying chicken was thoroughly ground and processed as described for beef. The respective moisture and fat contents of the ground, dried product were approximately 3 and 15%.

Carrots, precooked, freeze-dried. Carrots were cleaned, blanched, and sulfited. After being precooked for 15 min they were freeze-dried in a Vacudyne vacuum-freeze dehydration plant. Final plate temperature was 49°C; time of freeze-drying, 18 hr. Moisture content of the finished product was 1.1%.

Nitrogen. Water-pumped nitrogen, Air Reduction Company.

Helium. Grade A, National Cylinder Gas Company.

Nitrous oxide. U.S.P., Ohio Chemical Company.

RESULTS AND DISCUSSION

Magnitude of molecular oxygen values. In common with indirect methods (Haller and Holm, 1947; Lea *et al.*, 1943), the quantity of oxygen measured by the polarographic method is the fraction of the total quantity that is released to the surrounding medium. In this instance the electrolyte solution provided that medium.

The values reported below range from 0.05 to 6.0μM of oxygen per gram of sample. The values by all methods were in general agreement, although those obtained with the platinum electrode were somewhat higher (Table 1). The higher values may have been partly due to some diffusion of air into the solution. Literature values were not available for direct comparison, but some comparisons were possible. Haller and Holm (1947) reported oxygen values of 0.011–0.033 ml (approximately 0.4–1.3 μM) per gram of dried milk. The gas was desorbed from the dried milk by evacuation for 6 hr at 70°C. Karsten (1939) found 0.27μM oxygen per gram of garden-type loam (10% moisture) by the polarographic method. Emmett *et al.* (1938) found that air-dried Barnes soil adsorbed 1.0μM of oxygen per gram at 0°C at a partial pressure of 150 mm of oxygen. The soil was thoroughly degassed before oxygen was admitted to their manometric system.

Although molecular oxygen values were low, they were of the same order of magnitude as peroxide values, to which considerable importance is attached. It is frequently specified that the peroxide values of

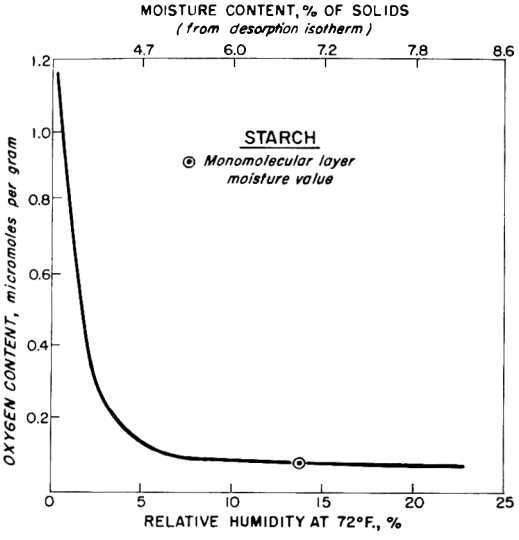

Fig. 1. Effect of desiccation on the molecular oxygen content of starch. Oxygen content by Winkler method and by dropping mercury electrodes.

fats and oils be controlled during refining, in the range of 1–5 mM per kg (or μM/g) to assure optimum quality (Williams, 1950).

Factors controlling the quantity of molecular oxygen. Moisture content. Fig. 1 shows the effect of desiccation on the oxygen content of starch. With a stable product such as starch, where no consumption of oxygen by chemical reaction would be expected under these conditions, oxygen values increased rapidly in the moisture region below the monomolecular-layer value (Salwin, 1959). This relation between oxygen content and moisture content was demonstrated consistently over several trials, using the dropping mercury electrode and the Winkler methods. Fig. 2 also shows that the oxygen content of beef was higher at moisture contents below the monomolecular-layer value.

Monomolecular layer values in Fig. 2 and in Table 1 exceed average values for freeze-dried meat processed in large-scale equipment by approximately 4% relative humidity. Laboratory freeze-dried meat had greater moisture-holding capacity, which resulted in higher monomolecular-layer values.

Karsten (1939) reported an inverse relation between oxygen content and moisture content of soil samples. Haller and Holm (1947) showed that the sorbed gas content of dried-milk samples decreased abruptly when moisture content exceeded 4%. They did not determine the possible influence of moisture content on their ability to remove sorbed gases by evacuation. The moisture content of the samples is not likely to be a factor influencing the technique reported here, because all samples were dispersed in water to displace the oxygen.

Once oxygen was bound to the three dehydrated beef samples at 0% relative humidity (Fig. 2), it was not possible to displace it by exposing the samples again to an air atmosphere of 23% relative humidity. The oxygen content of the very low-moisture samples remained at their maximal levels after such a transfer. After oxygen had become bound to charcoal at room temperature, it was impossible to desorb it by evacuation (Adamson, 1960). Such behavior emphasizes the desirability of excluding oxygen from dehydrated foods during processing.

The data in Table 1 show the relationship of oxygen content to the moisture con-

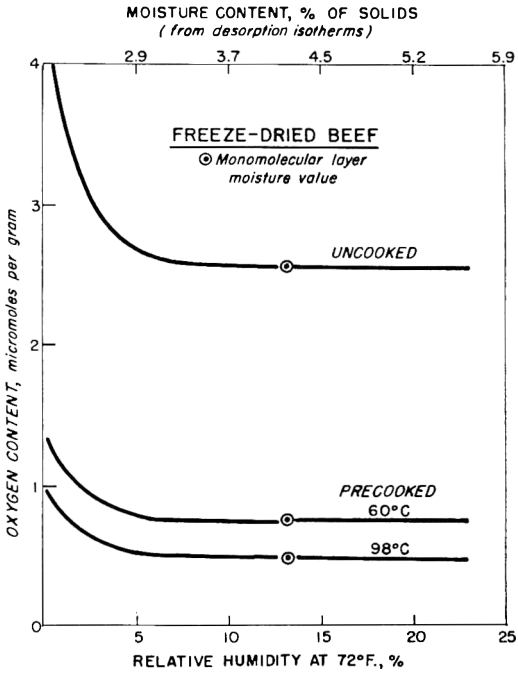

Fig. 2. Effect of desiccation on the molecular oxygen content of freeze-dried beef. Oxygen contents determined with polyethylene-covered platinum-silver electrode.

Table 1. Effect of processing and moisture content on molecular oxygen content of freeze-dried chicken (Sargent Model XXI polarograph).

Treatment	Bulk volume (cc/g)	Oxygen content, μmols/g			
		Platinum electrode		Dropping mercury electrode	
		0% RH (0.5% H_2O)	23% RH (5.4% H_2O)	0% RH (0.5% H_2O)	23% RH (5.4% H_2O)
Uncooked	11.4	3.7	4.7	2.4	3.6
Precooked 60°C	6.0	1.3	1.0	0.7	0.8
Precooked 98°C	5.5	0.9	1.1	0.6	0.8

Monomolecular layer value = 4.1% H_2O (13.7% RH).

tent of freeze-dried chicken. This product showed lower oxygen values upon desiccation below the monomolecular-layer moisture value of 4.1%. Fig. 3 shows the same relation between oxygen content and moisture content for freeze-dried carrots. At low moisture content the oxygen was apparently consumed by reaction with the unsaturated fat in these products. When the carrots were stored in air at room temperature, the oxygen contents decreased after the eleventh day (Fig. 3). They decreased more rapidly when the moisture content was below the monomolecular layer value. During the same period the carotene content of the low-moisture samples decreased, and the color faded. Apparently responsible for this further decrease in measurable oxygen was oxidation of the carotene.

The observations on these four products indicate that relatively stable materials exhibit a normal behavior of containing greater quantities of oxygen at lower moisture contents. A reversal in this pattern suggests that the sample under test is a less stable material in which oxygen is being consumed by fats, pigments, or other components. This hypothesis is consistent with observations that low moisture levels are frequently conducive to oxidation (Lea, 1958).

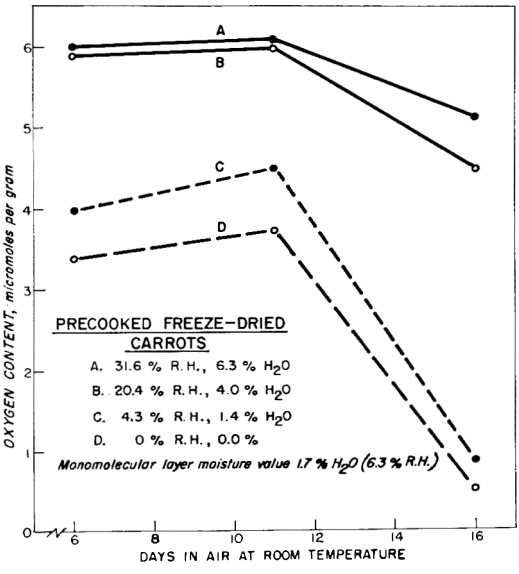

Fig. 3. Effect of desiccation and storage on the molecular oxygen content of precooked freeze-dried carrots. Note: Sulfite ion in neutral solution reduces oxygen rapidly (Kolthoff and Lingane, 1952). However, the sulfite concentration as determined by the polarographic method of Prater *et al.* (1944) was only 35 ppm, and the pH of the electrolyte suspension was 5.6.

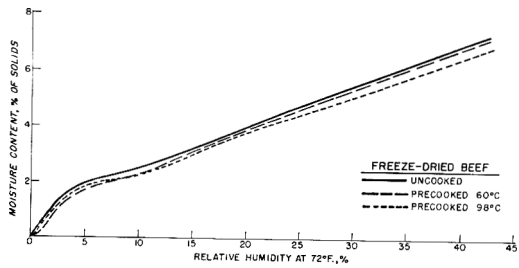

Fig. 4. Moisture adsorption isotherms of freeze-dried beef.

Processing. Fig. 4 shows that uncooked freeze-dried beef and beef precooked to internal temperatures of 60°C and 98°C had very similar moisture-sorption properties. Based on judgment formed from previous work in this laboratory (Salwin, 1959; Salwin and Slawson, 1959; Slawson and Salwin, 1958), the differences in moisture contents at a given relative humidity were not considered significant. On the other hand, the oxygen contents of freeze-dried beef were definitely lower when precooked (Fig. 2). The oxygen contents were apparently related to surface areas because the bulk volumes were: uncooked, 12 cc per gram; precooked to 60°C, 7 cc per gram; and precooked to 98°C, 6 cc per gram. The surface area controlled the oxygen content but not the moisture content. The same observations were made with freeze-dried chicken (Fig. 5 and Table 1).

These observations are consistent with those of Benson and Ellis (1948) and Benson and Richardson (1955). They reported similar findings for native and denatured egg albumin. They concluded that the adsorption of water by proteins is a different process from the adsorption of oxygen or nitrogen, and that the protein particle presents no internal surface accessible to either the oxygen or nitrogen molecule. They considered it highly probable that water adsorption is closely related to

hydration of specific polar regions in the individual protein moelcules rather than nonspecific adsorption on the surfaces of large aggregates. They hypothesized that the water molecule must actually spread apart layers of protein to penetrate to the polar groups.

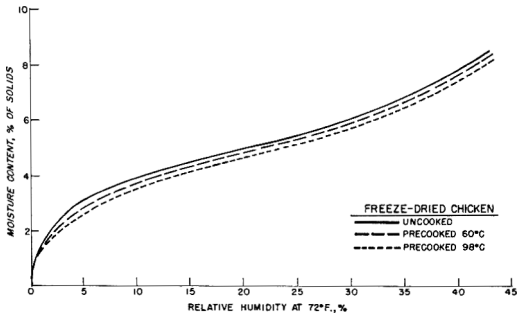

Fig. 5. Moisture desorption isotherms of freeze-dried chicken.

Headspace gas composition. Since gases have different adsorbabilities on solids, their competitive effect on the adsorption of oxygen was tested. The adsorbability of oxygen is intermediate between the high adsorbability of nitrous oxide (Bancroft, 1932) and the negligible adsorbability of helium (Adamson, 1960). Potato starch, conditioned to 0% and 1.25% relative humidity, was sealed in tin cans in oxygen-helium atmospheres and in oxygen–nitrous oxide atmospheres. The oxygen concentrations were 2, 10, and 20%. Included for comparison were air-packed samples (approximately 21% oxygen, 79% nitrogen). After 3 weeks at room temperature, the oxygen content of the starch was determined polarographically. In every case the oxygen contents were lower in the samples sealed with nitrous oxide and highest in those sealed with helium. Oxygen values were also lower at 1.25% relative humdity than at 0% relative humidity (Fig. 6).

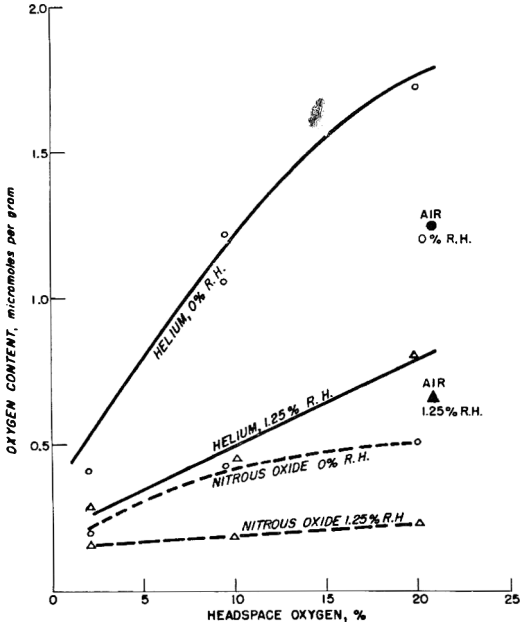

Fig. 6. Effect of headspace gas composition on the molecular oxygen content of starch.

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Studies on the Kinetics of Lipoxidase Inactivation Using Thermal and Ionizing Energy[a]

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(Manuscript received December 4, 1961)

SUMMARY

Lipoxidase demonstrated a typical protein inactivation response to heat as a function of pH when heated at 65°C and 0.1 molar buffer. The enzyme was found to be very sensitive to ionizing energy when irradiated in pH 7 buffer. This sensitivity was shown by a relatively low D_0 value (9×10^4 rad) and a rapid loss of enzyme activity during post-irradiation storage. The results of combined treatment of the enzyme, in buffer, by heat and radiation showed that treatment order was important. Heating prior to irradiation produced inactivation proportional to the sum of the separate treatments, whereas the reverse order produced inactivation greater than that calculated from the effect of each treatment. This is in agreement with the above, where post-irradiation storage at 0°C may be likened to a mild heat treatment. Addition of 20% pea solids to a buffered solution of the enzyme afforded a six- to tenfold protection with respect to inactivation by heat, and a seventyfold protection against inactivation by ionizing radiation. Combined heat and irradiation treatment of soybean lipoxidase, in the presence of 20% pea solids, showed that, in contrast to the results obtained in buffer, heat prior to irradiation produced greater enzyme inactivation than the reverse order of application of the two types of energy.

INTRODUCTION

The trend toward the use of relatively mild heat treatments in combination with physical or chemical techniques for preserving food materials has emphasized a newer problem relating to the loss of quality that can take place in fresh food materials through the action of enzymes in the food.

Amounts of thermal energy sufficient to destroy microbial contamination are generally adequate to inactivate all enzymes present in the food. Newer high-temperature short-time processes, however, present problems in relation to enzyme regeneration (Guyer and Holmquist, 1954).

The inhibition of food spoilage organisms by freezing, drying, antibiotics, chemicals, or ionizing energy leaves the enzyme systems in the food generally unaltered. In some cases degradation is actually hastened by these catalysts, through concentration of the enzyme and substrate and through cell breakdown during the preservation process.

The work reported herein was undertaken to determine the combined effects known to cause deterioration in some foods (Wagenknecht and Lee, 1958). These studies also had as an objective the determination of ways of optimizing processing conditions for enzyme inactivation with these forms of energy. The literature also shows that exact data are lacking on the inactivation kinetics of lipoxidase by thermal and ionizing energy. Therefore, the experimental work was divided into five parts:

1) The effect of heat on lipoxidase suspended in buffer at pH values of 4–9.

2) The effect of heat on lipoxidase suspended in pea puree.

3) The effect of irradiation treatment on lipoxidase suspended in pH 7 buffer and in pea puree.

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4) The combined effects of heat followed by irradiation treatment, and treatment in the reverse order, on lipoxidase suspended in pH 7 buffer.

5) The combined effects of heat followed by irradiation, and the reversal of the treatments, on lipoxidase suspended in pea puree.

The literature on each of the five phases of this study is quite extensive, especially on the effect of temperature and pH on the rate of loss of physiological activity of various proteins and enzyme systems. [The reader is referred to Laidler (1958), Reiner (1959), Levy and Benaglia (1950), and London *et al.* (1958) for a general discussion of the effects of temperature and pH on the rate of loss of physiological activity of protein.]

Fricke (1952) and Leone *et al.* (1959) have discussed the combined effects of thermal and ionizing energy on crystalline egg protein. The results obtained in the present study, with soybean lipoxidase, are similar to those found by the above workers. The implications of our results on the preservation of food appear in the discussion section.

EXPERIMENTAL

Procedures. *Assumptions.* The following assumptions were made: 1) a first-order rate of inactivation of the enzyme with respect to time of heat treatment; 2) absolute rate theory for the kinetics of lipoxidase inactivation by thermal energy (Glasstone *et al.*, 1941).

Enzyme preparation. Lyophilized purified soybean lipoxidase was used, a single one-g sample, held at −18°C, being sufficient for all experimental purposes. Assay by the method of Bergstrom and Holman (1948) showed that the enzyme preparation had an activity of about 4.4% that of the crystalline lipoxidase prepared by the above workers. Measurement of optical density at 280 and 260 mμ (Colowick and Kaplan, 1955) indicated that the Worthington preparation (Worthington Biochemical Co., Freehold, N. J.) was about 50% protein.

Stock enzyme. Stock solutions of the enzyme, at a desired level of activity, were prepared by dissolving a weighted quantity of the lyophilized preparation in chilled 0.1*M* buffer of the appropriate pH. These solutions at 5°C were found to be sufficiently stable for use over a 24–48-hr period, though a control was always run to check activity. Since the enzyme was not crystalline pure, all activity measurements were based on a weight concentration of the preparation, the assumption being that the activity measured on a weight basis would differ only from the activity measured on a molecular-weight basis by a constant factor.

The concentration of lipoxidase in the standard stock enzyme solution was about 0.3 mg of the lyophilized preparation per ml. Individual samples were prepared by dispensing 0.03 ml of the stock solution, from a 1-ml syringe fitted with a micrometer, into thin-walled melting-point capillary tubes. Each tube, containing about 9 μg of the enzyme preparation, was sealed with an oxygen-gas flame and stored at 0°C until assayed.

Enzyme in pea solids. Enzyme preparations containing pea solids were made as follows. Dried split peas (about 8% moisture) were ground in a Wiley mill and sifted through an 80-mesh sieve. One g of the resultant flour, free of cotyledons, was mixed with 5 ml of 0.05*M*, phosphate-citrate buffer, pH 7, containing 0.6–0.7 mg of lyophilized lipoxidase per ml. The final pH was 7.0 and the final concentration of pea solids was 20% by weight. Capillary tubes were filled with 0.015 ml of the puree to give a final enzyme concentration of 7 μg of lipoxidase preparation in each tube. A comparison of the activity of this preparation with tubes containing 7 μg of lipoxidase and no pea solids showed that this preparation had about 70% of the activity of the enzyme in pH 7 buffer. All activities were expressed in terms of controls run with each experiment. These controls were run to compensate for small variations in activity from preparation to preparation.

Substrate preparation. The substrate used throughout was made from 99% pure linoleic acid (Hormel Foundation, Austin, Minnesota) held at −18°C in a nitrogen atmosphere under reduced pressure.

Substrate solutions were prepared fresh each day by weighing 50 mg of linoleic acid into a volumetric flash and adding sufficient 0.1*M* sodium borate-boric acid buffer, pH 8.9, to give a substrate concentration of 1 mg/1 ml. Tween 20 (0.3 mg/ml), added as an emulsifier, did not appear to interfere with the activity of the enzyme at the concentrations used in the assay solution. The concentration of the linoleic acid in the stock solution was 3.57m*M*.

Buffer preparation. Buffers, of a modified McIllvain type (Hodgman, 1951), were prepared by mixing 0.1*M* disodium phosphate (Na_2HPO_4) with 0.1*M* citric acid to the desired pH as shown with a Beckman Model H-2 glass-electrode pH meter. Borate buffers were prepared by mixing 0.05 moles of sodium borate with 0.05 moles of boric acid in 1 L of distilled water.

Enzyme assay technique. A spectrophotometric

method, adapted from Tappel *et al.* (1952), was used for all assay work. The following procedure was selected after a preliminary investigation of the effect of substrate and enzyme concentration on the rate of the reaction.

A capillary tube, containing about 9 μg of enzyme preparation in 0.03 ml of buffer, was placed in 2.7 ml of aerated 0.1*M* borate buffer, pH 9, contained in a heavy-walled 16 × 139-mm test tube. The capillary tube was crushed with a glass stirring rod, and the solution was thoroughly mixed. The contents of the test tube were poured directly into a 4-ml one-cm-light-path silica Beckman UV spectrophotometer absorption cell. At zero time, 1 ml of substrate was added with a rapid-flow pipette, and the cell was covered with a glass cover and then inverted to mix enzyme and substrate solution.

Optical density (at 234 mm) of the reaction mixture was recorded at 30-sec intervals and plotted against time on rectangular graph paper. The slope of the straight-line portion of the activity curve was found to be proportional to enzyme activity. This procedure gave results equal to those of the Bergstrom-Holman method, cited earlier.

A typical value for the activity of the unheated enzyme preparation, at a concentration of 2.43 μg per ml of final reaction mixture, was 0.600 OD per min. To simplify the handling of data, all activities were multiplied by 1000 to eliminate the decimal point. Thus, the range of activities measurable with reasonable accuracy (plus or minus 3%) was about 10–600 units (0.010–0.600 OD/min). Fig. 1 shows three such activity curves and a plot of enzyme concentration versus activity.

A modification of this technique was used to assay soybean lipoxidase in the presence of pea solids. In this case capillary tubes were crushed in 2.7 ml of borate buffer containing about 10–20 mg of Hyflow Super Cell filter aid (Johns-Manville Celite). The thoroughly mixed contents were filtered by mild suction through a sheet of STS No. 595 filter paper supported by a 16-mm Buchner funnel, directly into a 1-cm Beckman UV absorption cell. Reaction temperature was about 20°C.

Heating technique. The heating apparatus used, a modication of that developed by Stern and Proctor (1954), is shown in Fig. 2. Thin-walled melting-point capillary tubes with an OD of less than 2 mm were used throughout this work. Calculations showed that about 10 sec were required for the contents in the center of the tube to reach 0.1°C of bath temperature for a 75°C temperature rise. Corrections for heat-penetration lag were estimated by extrapolating the first-order rate-of-destruction curve obtained for heating times longer than 10 sec. In all cases, the activity of a treated enzyme preparation (N) was expressed as a ratio

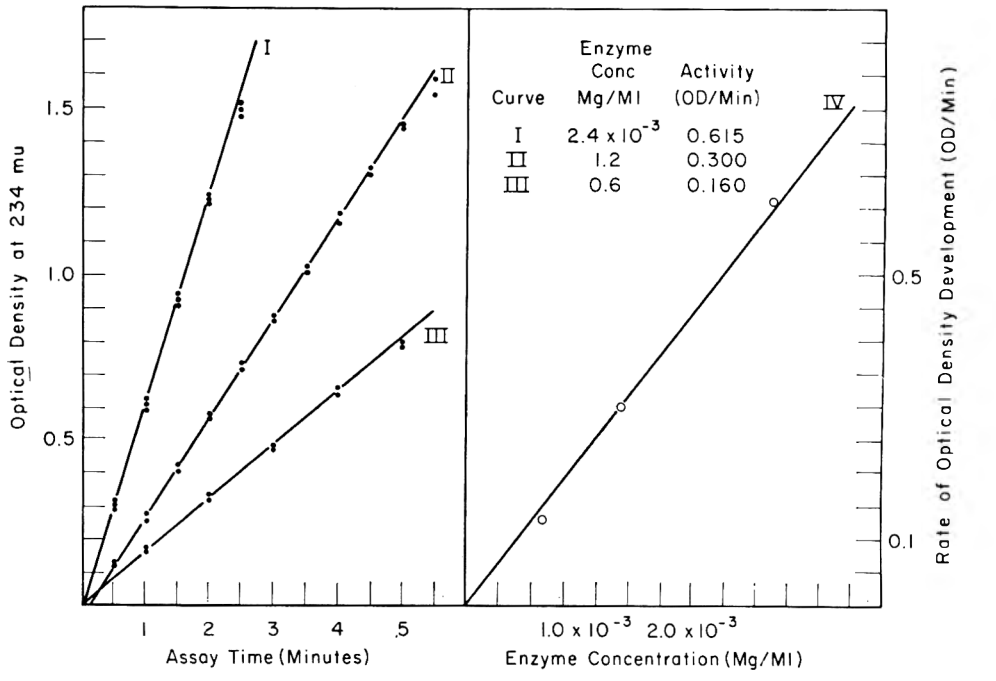

Fig. 1. Graphic technique used in the analysis of lipoxidase activity.

of that of an untreated control (N_o) assayed at the same time.

Irradiation technique. Irradiation was performed in the Co^{60} source described by Davison *et al.* (1953). The dose rate, as measured by ferrous sulfate dosimetry (Weiss *et al.* 1956), was about 165×10^3 rad per hr, and was corrected for decay with the factors appearing in Kinsman (1954). The temperature of samples exposed for short periods was held below 10°C by crushed ice. Samples exposed for longer periods approached the temperature of the source, 20°C. Details on the handling of the capillary tubes during experiments using both heat and irradiation treatments are given where appropriate.

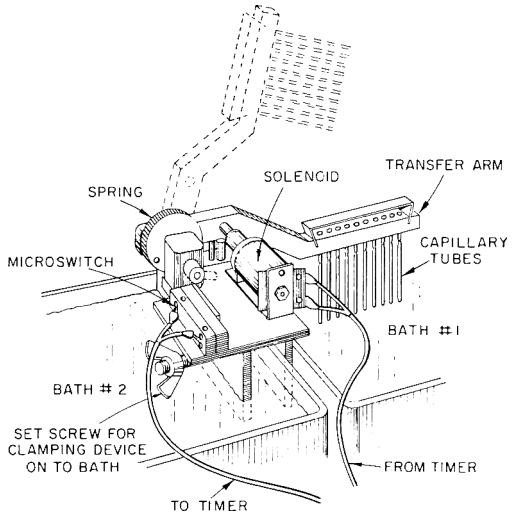

Fig. 2. Capillary tube transfer device.

EXPERIMENTAL DATA

Thermal and pH effects. The inactivation of lipoxidase by heat appeared to follow first-order kinetics down to residual enzyme activities of about 10% of the initial value. This was found to be true at all values of pH between 4 and 9, except pH 8. The linear relation between the logarithm of the residual activity and time of heating allowed the determination of the inactivation rate constant (sec^{-1}) by the method of least squares.

Fig. 3 presents a plot of the rate of inactivation constants so obtained as a function of pH for the inactivation of soybean lipoxidase at 65°C. The value shown at pH 8 represents an estimate based on an average of three runs. The dashed lines through the experimental values are drawn to indicate three possible regions for relation of the rate of inactivation of lipoxidase to pH (cf. Levy and Benaglia, 1950).

Fig. 4 presents an Arrhenius-type plot of the data for the rate of inactivation of lipoxidase in pH 7 and pH 4 buffer. Because of the sensitivity of lipoxidase toward heat at pH 4, the accuracy of these data represented by the three points obtained at this pH was not as great as at pH 7. Table 1 contains the thermodynamic values calculated from the data shown in Fig. 4.

Table 1. Thermodynamic values calculated for the inactivation of lipoxidase in pH 4 and pH 7 buffer.

pH	T°C	$\triangle H^*$ (Kcal/mole)	$\triangle S^*$ (Cal/deg-mole)	$\triangle F^*$ (Kcal/mole)
4	65	100	255	18.7
7	65	101	242	22.9

$\triangle H^*$ = activation energy.
$\triangle S^*$ = entropy of activation.
$\triangle F^*$ = free energy of activation.

The curves in Figs. 3 and 4 are representative of the data obtained from similar studies on ricin, prosthetic acid phosphatase, and other enzymes.

The effect of heat on preparations of lipoxidase suspended in 20% pea solids is shown in Figs. 5 and 6. Two regions of heat resistance were observed, lipoxidase becoming more sensitive to inactivation by heat above 70°C. Table 2 shows

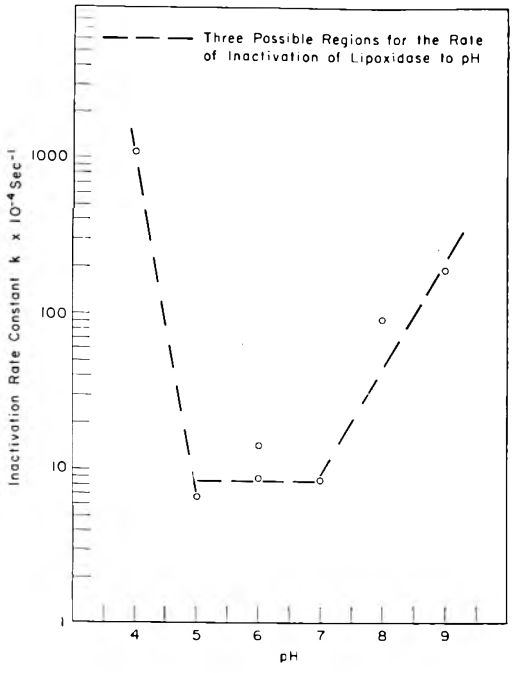

Fig. 3. Rate of inactivation of lipoxidase as a function of pH.

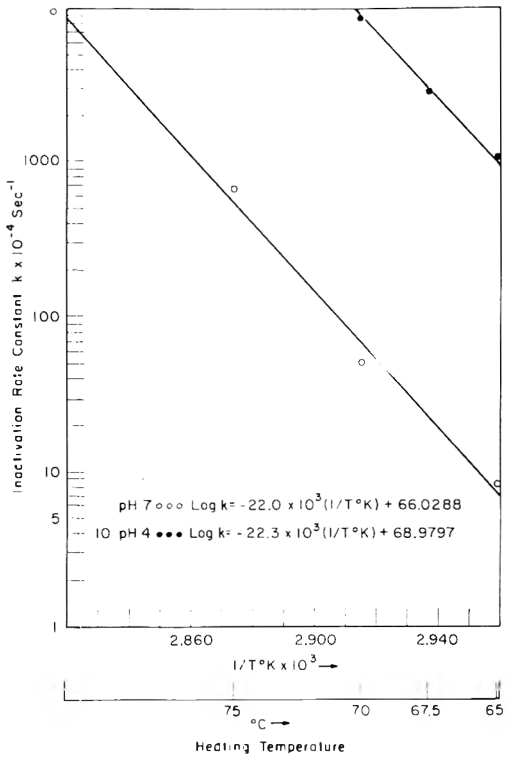

Fig. 4. Arrhenius type of plot of the rate of inactivation data for lipoxidase in pH 7 and pH 4 buffer.

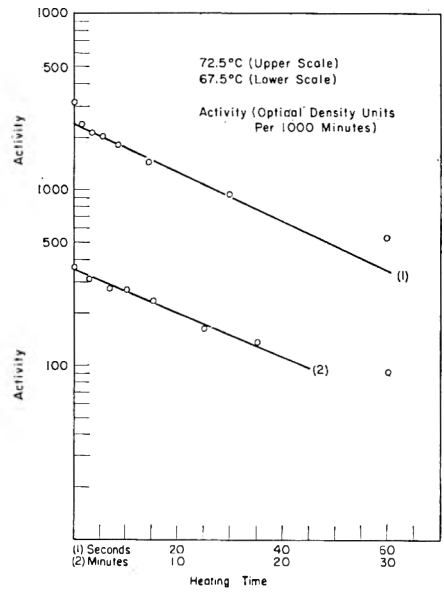

Fig. 5. Inactivation of lipoxidase in the presence of 20% pea solids at 67.5 and 72.5°C.

Table 2. Thermodynamic values calculated for the inactivation of lipoxidase in the presence of 20% pea solids.

Temperature Range	T°C	△H* (Kcal/mole)	△S* (Cal/deg-mole	△F* (Kcal/mole)
65–70°C	65	64	115	25
70–80°C	65	150	366	26

the effect of pea solids on the thermodynamic values obtained for the inactivation of lipoxidase.

Irradiation effects. The results obtained when lipoxidase in pH 7 buffer was exposed to ionizing radiation were quite unexpected in that as little as 25,000 rad was sufficient to inactivate 90% of the enzyme if assayed 24 hr after irradiation. Post-irradiation loss of activity is a common occurrence with biological materials, the phenomenon being the subject of a symposium (*cf.* Alper, 1952), and the implications of this loss of activity after treatment with a small dose of ionizing radiation will be discussed later. Because of the rapid loss of enzyme activity after irradiation, no curve could be obtained showing residual enzyme activity as a function of dose. However, if the rate of inactiva-

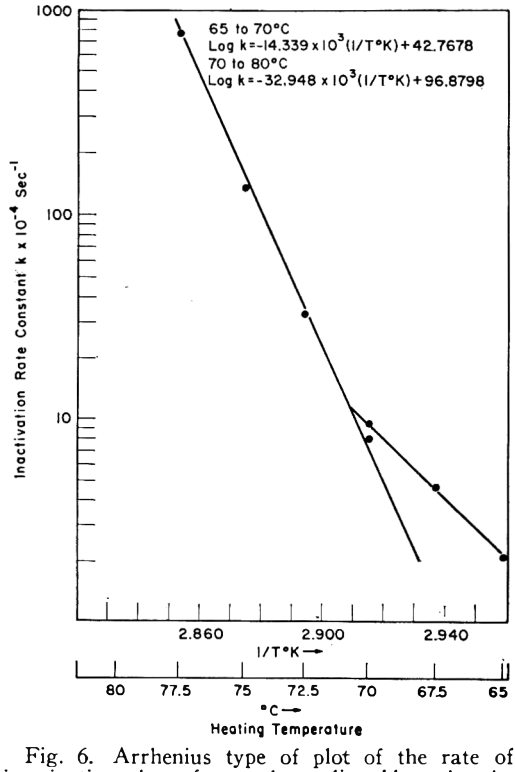

Fig. 6. Arrhenius type of plot of the rate of inactivation data for soybean lipoxidase in the presence of 20% pea solids.

tion curves for post-irradiation storage of the enzyme, assumed to be first order, were extrapolated to zero time (i.e., just out of the source), and the length of time of exposure in the source neglected, a D_0 (dose required to destroy 67% of the activity of a given concentration of the enzyme) of about 9×10^4 rad is obtained.

Irradiation of lipoxidase preparations in the presence of 20% pea solids greatly increased the resistance of the enzyme to inactivation, as would be expected. A D_0 value of 6.2×10^6 rad was found, indicating that the pea solids afforded about a seventyfold protection to the enzyme. Furthermore, irradiated preparations containing 20% pea solids showed no tendency toward loss of activity during post-irradiation storage for 24 hr at 0°C.

Combined effects of heat and irradiation treatment on the activity of lipoxidase suspended in pH 7 buffer. Lipoxidase, in pH 7 buffer, was quite sensitive to ionizing radiation. This sensitivity was shown both by a relatively low D_0 value (9×10^4 rad) and by a rapid loss of residual activity during post-irradiation storage. Table 3 shows the loss of enzyme activity during storage at 0°C for lipoxidase solutions irradiated at 25, 50, and 100 krad. Included is the effect on the residual activity of the enzyme of a post-irradiation heat treatment calculated to destroy about 25% of the activity of the unirradiated enzyme. The post-irradiation heat treatment of lipoxidase increased the lethal effect of the thermal energy by manyfold.

The effect of post-irradiation heat treatment on the activity of lipoxidase was studied in a more quantitative manner by using a dose of 10^4 rad (3.6 min exposure) followed by a short heat treatment at 65 or 70°C. In these studies, the enzyme preparation was filled into capillaries and irradiated. The irradiated capillaries were heated for the appropriate times and temperatures, and removed to 0°C storage until assayed. Table 4 shows the effect of heat treatments calculated to destroy about 2–25% of the activity of the unirradiated enzyme. A dose of 10^4 rad gave a negligible amount of inactivation over a post-irradiation storage period of 40 min. A heat treatment of 30 sec and 65°C (sufficient to inactivate 2% of the unirradiated enzyme) caused a 63% inactivation when the treated samples were assayed within 1 hr of exposure.

The post-irradiation loss of activity of solutions of lipoxidase appeared to be of sufficient interest to merit detailed study on residual activity. The objective was to determine the effect of storage

Table 3. Effect of irradiation and storage at 0°C on lipoxidase activity in pH 7 buffer.

Treatment	Post-irrad. storage at 0°C (min)	Average activity [a]	Percent residual activity
Control (no treatment)		615	100
Irradiated 25×10^3 rad	60	175	28.5
(9-min exposure)	70	170	27.5
	85	150	24
	1530	63	10
Irradiated 50×10^3 rad	27	175	28.5
(18-min exposure)	42	165	27.0
	110	115	18.5
	1530	52	9.5
Irradiated 10^5 rad	140	51	8.3
(36-min exposure)	150	68	11.0
	170	50	8.1
	185	47	7.6
Irradiated 2×10^5 rad	140	38	6.2
(73-min exposure)	150	38	6.2
	160	22	3.6
	170	30	4.9
Irradiated 10^5 rad, then	220	Trace	Calculated-found
heat 50 sec @ 70°C	220	Trace	22% Trace
Irradiated 2×10^5 rad, then	240	Trace	7% Trace
heat 50 sec @ 70°C	240	Trace	

[a] Activities expressed in optical density units per 1000 min.

Table 4. Effect of irradiation and post-irradiation heat treatment on soybean lipoxidase in pH 7 buffer.

Treatment	Storage at 0°C (min)	Average activity [a]	Percent residual activity	
			Found [b]	Heat only
Control (no treatment)		600	100	
Irradiated 10^4 rad	30	615	100	
	35	595	100	
	40	570	95	
Irradiated 10^4 rad followed by heat at 65°C for the times noted				
30 sec	0		(76%)	98%
	50	225	37.5	
	60	220	37	
60 sec	0		(50%)	95%
Half value 64 min	25	250	42	
	75	140	23	
	125	95	16	
120 sec	0		(36%)	90%
Half value 54 min	15	180	30	
	65	90	15	
	100	60	10	
180 sec	0		(21%)	86%
Half value 64 min	85	51	8.5	
	115	36	6.0	
	140	28	4.7	
Irradiated 10^4 rad followed by heat at 70°C for the times noted				
24 sec	90	260	43	87%
	110	255	43	
60 sec	60	68	11	75%
	75	66	11	

[a] Activities expressed in optical density units per 1000 min.
[b] Values in parentheses are extrapolations of storage data to zero storage time based on first-order loss of activity during storage.
Half values shown are for post-irradiation loss of activity upon storage at 0°C based on first-order kinetics.

at 0°C and heat at 70°C. The assumption was that the combined effects of post-irradiation heat treatment and irradiation would be independent of treatment time when the activities of the enzyme preparation were corrected for activity loss during storage. Samples were assayed for residual activity after storage at 0°C for up to 3 hr; Table 5 shows the results. Therefore, tubes of lipoxidase, in pH 7 buffer, were subjected to the following treatments: 1) irradiation with 5000 rad; 2) irradiation with 5000 rad followed by a heat treatment of 30 sec at 70°C, given at 8, 68, 128, and 140 min after irradiation; and 3) heat treatment for 30 sec at 70°C.

Table 5 shows that, within experimental error, the residual activity of irradiated lipoxidase is fairly independent of the time the enzyme is heated after irradiation, but is strongly dependent on the length of time between irradiation and assay. Finally, lipoxidase preparations in pH 7 buffer were heated for 30 sec at 70°C before irradiation in order to observe any effects, due to the presence of heat-denatured enzyme, on the rate of post-irradiation loss of activity of lipoxidase. The data thus obtained were best expressed by a first-order rate curve having the following constants: half value = 165 min; $N_0 = 473$ (81%) (at zero storage time); Log $N = -1.825 \times 10^{-3}t + 2.6746$. The rate of loss of activity of lipoxidase after irradiation appeared independent of the order of treatment. The magnitude of residual enzyme activity appeared to be less when heat treatment followed irradiation of the enzyme at this dose level. Fig. 7a plots data in Tables 4 and 5.

Similar studies were carried out at the 10^4 and 2×10^4 rad dose level, with and without pre- and

Table 5. Effect of post-irradiation heat treatment and storage at 0°C on the residual activity of lipoxidase in pH 7 buffer.

Treatment	Post-irradiation storage time (min)	Post-heating storage time (min)	Residual [a] activity	Percent residual activity
Control (no treatment)	.....	.....	585	100
Heated 30 sec at 70°C	.....	.....	550	94
Irradiation dose 5000 rad				
(no heat treatment)	20	.....	505	86.5
	30	.....	505	86.5
	40	.....	450	77
	55	.....	450	77
	110	.....	390	67
	120	.....	380	65
Irradiation dose 5000 rad, followed				
by heat treatment for 30 sec at 70°C	13	5	400	68.5
	23	15	410	70
	70	3	315	54
	80	70	315	54
	128	1	250	43
	138	70	250	43
(three samples)	145, 155, 170	5, 15, 30	250, 250, 240	43, 43, 41
	151	143	240	41
	187	60	195	33

[a] Percent residual activity based on a control activity of 585 units.

Activities expressed in optical density units per 1000 min.

The rate of loss of activity of the samples irradiated at 5000 rad without heat treatment could be described as first-order rate curve with the following constants: Half value: 217.1 min. N_o Calc.=533 (91%) (at zero storage time) Log $N = -1.3863 \times 10^{-3}t + 2.7271$, while the data for the post-irradiation heat-treated samples followed a curve described as: half value: 167 min. N_o calc. = 434 (74.1%) (at zero storage time) Log $N = -1.803 \times 10^{-3}t + 2.6377$.

Table 5a. Effect of a pre-irradiation heat treatment of 30 sec at 70°C on the residual activity of lipoxidase exposed to 5000 rad of gamma radiation 12 min after heating.

Treatment	Post-irrad storage time at 0°C (min)	Activity	Percent residual activity	Data from Table 5	
				% activity	Storage time (min)
Control	.....	640	100%		
Heated 30 sec at 70°C		565	96.5		
Irradiated, 5000 rad	30	490	83.8	86.5%	30
	90	360	61.5	70 (estimate)	90
Heated 30 sec at 70°C and irradiated with 5000 rad					
1	15	435	74.4	68.5	13
2	24	435	74.4	70	23
3	71	350	60.0	54	70
4	80	340	58.0	54	80
5	145	260	44.4	43	123
6	158	240	41.0	43	138
7	218	170	29.0	41	151
8	228	170	29.0	33	187

Half value = 165 min $N_o = 473$ (81%) Log $N = 1.825 \times 10^{-3}t + 2.6746$

Activities expressed in optical density units per 1000 min.

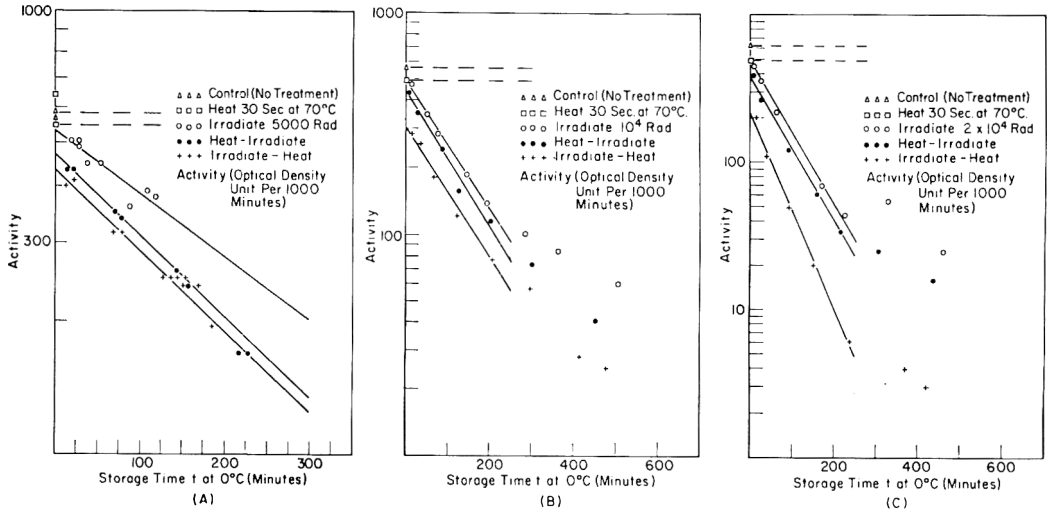

Fig. 7. Loss of activity of lipoxidase stored at 0°C after a combined heat and irradiation treatment.

post-irradiation heat treatment. Figs. 7b and 7c show the results, and Table 6 summarizes the constants, describing the rate curves obtained. Table 6 indicates the interaction between treatment order and dose level as shown by the post-irradiation rate of loss of activity of the enzyme.

DISCUSSION

The effect of thermal energy on proteins in solution has been extensively discussed by Kauzmann (1954), Laidler (1958), Levy and Benaglia (1950), London *et al.* (1958), and others. Their findings are applicable to the results obtained for the heat treatment of lipoxidase.

This discussion describes and evaluates a mechanism whereby the data presented may be explained and correlated.

Lipoxidase, in dilute buffer solution, may be pictured as an ordered protein molecule, partially hydrated (*cf.* Klotz, 1958), and possessing the proper hydrogen bonds to maintain itself in its active form. Water of "hydration," pictured as surrounding the molecule in dilute solution, may act as a

Table 6. Summary of constants describing the rate curves for the post-irradiation loss of activity of lipoxidase held at 0°C in pH 7 buffer.[a]

Treatment	Half-value [b] (min)	Calculated N_0 value % [c] (zero storage time)	Equation of curve [d] log N =
5000 rad	217	91	$-1.386 \times 10^{-3}t + 2.727$
5000 rad then heated [e]	167	74	$-1.803 \times 10^{-3}t + 2.638$
Heated then 5000 rad	165	81	$-1.825 \times 10^{-3}t + 2.675$
10^4 rad	104	86	$-2.900 \times 10^{-3}t + 2.685$
Heated then 10^4 rad	98	77	$-3.068 \times 10^{-3}t + 2.635$
10^4 rad then heated	101	52	$-2.997 \times 10^{-3}t + 2.477$
2×10^4 rad	61	77	$-4.925 \times 10^{-3}t + 2.674$
Heated then 2×10^4 rad	61	61	$-4.943 \times 10^{-3}t + 2.573$
2×10^4 rad then heated	46	34	$-6.579 \times 10^{-3}t + 2.315$

[a] These data are based on storage times of less than 300 min.
[b] Half value means the storage time at 0°C necessary for the preparation to lose 50% of its initial activity.
[c] Percent activities are based on untreated controls run simultaneously with each experiment. See Fig. 7a,b,c for values.
[d] Activities represented by the equations are in optical density units per 1000 min.
[e] Heat treatment consisted of 30 sec at 70°C.

stabilizing agent through increasing hydrogen bonding.

Lipoxidase, suspended in 20% pea puree, is also pictured as an ordered enzyme, but it is postulated that the enzyme exists as a partial complex with some material present in the puree. Neither the nature of the complexing material nor the complex was determined, but investigators have shown, for example, that a protein fraction of wheat will complex with amolytic enzymes and thus partially inhibit their activity (Kneen and Sandstedt, 1946).

Evidence of complex formation. The idea of complex formation is neither new nor unique. Whenever an enzyme is purified, substances present in the starting material are removed, and with each subsequent removal an increase in activity noted. Because biological materials are so heterogeneous, the form of the enzyme-"surrounding media" complex may partially take the form of a true chemical complex possessing a true equilibrium constant, or the binding of the enzyme could possibly be pictured as an entanglement of the molecule in a starch or protein matrix. Addition of a known concentration of lipoxidase to a volume of pea puree resulted in a loss of about 30% of the activity of the enzyme, based on its activity in buffer. This loss of activity in the presence of pea puree may be correlated in a rough manner with the change in the heat resistance of the enzyme in the absence and presence of this protective substance.

Tables 1 and 2 show the thermodynamic data obtained for lipoxidase heated in buffer and in pea puree. A change in the pH of the suspending buffer was found to change the entropy of activation ΔS^*. Thus, as the nature of the buffer is changed, becoming either more alkaline or acid than pH 7, it presumably became easier and easier to form an activated complex, by heat, through which the enzyme could inactivate. As a corollary, any material that would prevent or inhibit the formation of this activated complex would inhibit inactivation of the enzyme and thus protect it from heat. The addition of pea puree was found to increase the resistance of lipoxidase to heat inactivation although the thermodynamic values associated with this increase in heat resistance did not indicate the nature of the protection.

For example, London *et al.* (1958) studied the effect of prostatic acid phosphatase inhibitors on the heat resistance of this enzyme and found that the degree of inhibition could be correlated with the amount of protection afforded. The protective mechanism appeared to involve the straddling of a critical seam of the enzyme molecule by the inhibitor. This auxiliary binding of the protein molecule by the inhibitor was sufficient to increase the resistance of the protein to heat.

Through a calculation similar to that carried out by London *et al.* (1958) the loss in activity upon the addition of lipoxidase to pea puree could be shown to agree, within a factor of ten, to the protection afforded the enzyme against inactivation by heat.

Effect of treatment order, on residual activity in presence of 20% pea solids. A study was made of the effect of order of treatment on the residual activity of soybean lipoxidase in the presence of 20% pea solids. Such a study should supply data representative of processing conditions that might be encountered in the preservation of food by thermal and ionizing energy. Capillary tubes were filled, sealed, and stored at 0°C according to the procedures previously outlined.

Three studies were performed to determine the effect of treatment order, dose level, and heating temperature on the residual activity of the enzyme. Table 7 lists the treatments and variables used. Heating temperatures of 72.5 and 67.5°C were selected to represent inactivation conditions above and below 70°C since it appeared that the activation energy of inactivation of lipoxidase in the presence of pea solids was different above and below this temperature.

Typically, a run was carried out by preparing sufficient tubes to give four tubes per heating interval (40 tubes total), six untreated controls, and six tubes for irradiation treatment but without heat. No samples treated only with heat were run, since inclusion of this variable would have made the experiment prohibitively long (over 12

Table 7. Treatments and variables used to determine the effect of thermal and ionizing energy on lipoxidase activity in the presence of 20% pea solids.

Treatment	Temperature (°C)[a]		Irradiation dose (rad)	
	72.5 [b]	67.5 [c]	0.6×10^6	2.0×10^6
Heat-Irradiate	A	B,C	A,B	C
Irradiate-Heat	A	B,C	A,B	C
Heat only [d]	(A)	(B,C)		
Irradiate only			A,B	C

[a] Letters A, B, C refer to single runs.
[b] Heat treatments at 72.5°C were for 0.5, 1, 2, 3, and 5 min pre- and post-irradiation.
[c] Heat treatments at 67.5°C were for 7, 15, 25, 35, and 48 min pre- and post-irradiation.
[d] These data were taken from previous runs.

hr). All samples were filled at the same time and held in melting ice until treated and assayed.

Table 8 and Fig. 8a,b,c show the results. The curves drawn through the experimental points represent least-square fits to the average values exclusive of the points for zero heating times. The spread of experimental values is shown in each plot. It should be noted that the variation in activity was much greater for samples heated before irradiation than for samples treated in the reverse order.

Inspection of Fig. 8 shows that heat treatment after irradiation resulted in less inactivation of the lipoxidase, added to the pea puree, than the reverse procedure, but the *rate of lipoxidase inactivation* with heating appeared to be independent of irradiation dose. That is, after an initial rapid loss of activity the residual enzyme was inactivated in a manner similar to that observed for the heat-treated enzyme. The curves in Fig. 8, although drawn as if first order, are not to be taken as demonstrating the order of the reaction. At long heating times the order of the reaction, and consequently the rate, appeared to change, while for very short heating times inactivation occurred at a very great rate.

Results of the work outlined in this section are tabulated in Table 9 for reference.

Effect of thermal and ionizing energy on lipoxidase in buffer solution. The effect of ionizing energy on proteins in dilute solution appears to be chemical in nature. Radicals, formed in the bulk solution, have been shown to react with various groups on the protein molecule to cause both hydrogen and

Table 8. Effect of pre- and post-irradiation heat treatment on soybean lipoxidase in 20% pea puree.[a,b]

Treatment	Half value (sec)	Rate constant $k(sec^{-1})$	N_0 calc.	N_0 found	Equation [c] $\log N =$
A					
0.6×10^6 rad, 72.5°C					
Irradiate-heat	190	36.37×10^{-4}	330	480	$-1.58\times10^{-3}t + 2.52$
Heat-irradiate	189	36.61×10^{-4}	207	480	$-1.59\times10^{-3}t + 2.32$
B					
0.6×10^6 rad, 67.5°C					
Irradiate-heat	1604	4.32×10^{-4}	322	455	$-1.88\times10^{-4}t + 2.51$
Heat-irradiate	1655	4.19×10^{-4}	258	455	$-1.82\times10^{-4}t + 2.41$
C					
2.0×10^6 rad, 67.5°C					
Irradiate-heat	1570	4.40×10^{-4}	216	310	$-1.92\times10^{-4}t + 2.34$
Heat-irradiate	1595	4.33×10^{-4}	99	310	$-1.89\times10^{-4}t + 1.99$
Heat 72.5°C	215	32.24×10^{-4}	244	314	$-1.4\times10^{-3}t + 2.39$
Heat 67.5°C	1495	4.62×10^{-4}	342	360	$-2.0\times10^{-4}t + 2.53$
Irradiate 0.6×10^6 rad	A = 480	B = 455			
Irradiate 2.0×10^6 rad			C = 310		

[a] Activities expressed in optical density units per 1000 min.
[b] Untreated controls had the following activities: A-540, B-515, C-430.
[c] Activities represented by equations are in optical-density units per 1000 min.

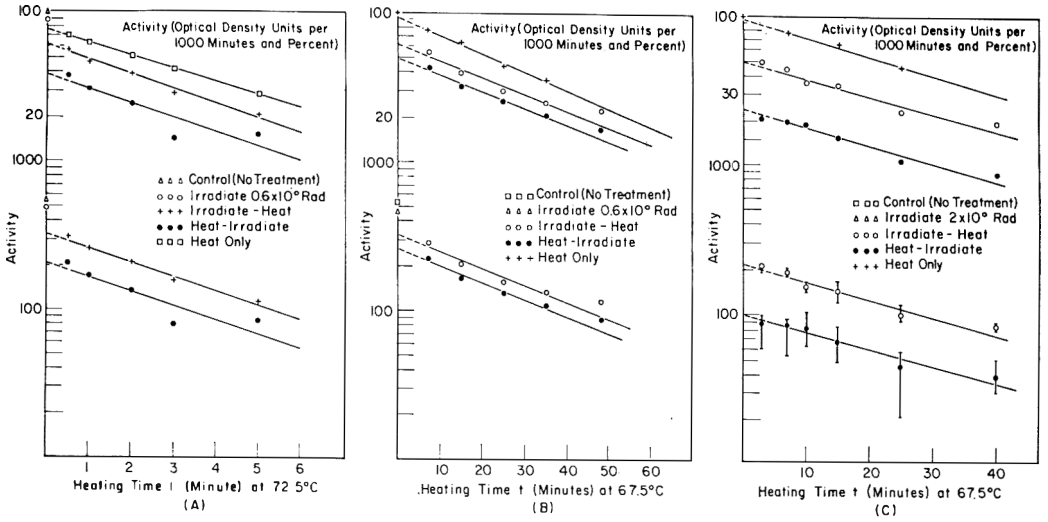

Fig. 8. Combined effects of heat and irradiation treatment on lipoxidase in the presence of 20% pea solids.

covalent bond breakage. Thus, the tertiary, or over-all, structure of the protein molecule, held together largely through hydrogen bonding, is weakened.

The initial loss of lipoxidase activity, as a function of irradiation dose, appeared to be first order and typical of many organic materials. That the tertiary structure of the enzyme may be weakened, is shown by the rapid loss of activity of the enzyme under post-irradiation heat treatment.

Heat treatment before irradiation inactivated a certain fraction of the lipoxidase molecules present in solution. It may be assumed that those not inactivated were otherwise unaltered by heating the preparation. This would be in agreement with the postulates of the absolute-rate theory since the active complex, which presumably decomposes to form inactive enzyme, may be assumed to be in true equilibrium with the native enzyme.

Irradiation of the remaining active enzyme caused a loss of activity proportional to the concentration of the active enzyme remaining after the mild heat treatment. When the reverse treatment was employed (irradiation followed by heat), the irradiation-weakened tertiary structure of the enzyme collapsed rapidly under the effects of heat. The treatment of irradiated enzyme with heat hastened the breakdown that was found to occur during storage. In effect, irradiation greatly reduced the activation energy for inactivation of the enzyme.

Table 9. Tabulation of the residual activity percent obtained by extrapolating various rate-of-destruction curves to zero heating time.

Irradiation dose heat treatment	zero	0.6×10^6	2.0×10^6
None	100	88.5	72.1
67.5°C	95.1		
Irradiate-heat		62.5	
Heat-irradiate		50.0	23.0
Calculated as additive		84.0	68.5
72.5°C	77.6		
Irradiate-heat		61.2	
Heat-irradiate		38.4	
Calculated as additive		68.6	

(Data taken from Fig. 8a,b,c.)

The effect of thermal and ionizing energy on lipoxidase in the presence of 20% pea solids. The results obtained through application of thermal and ionizing energy to lipoxidase in the presence of pea puree appeared to be in direct contrast with those obtained by treating the enzyme in buffer solution. A plausible explanation has been developed based on the assumption that the

lipoxidase, added to the pea puree, existed partially in a puree-enzyme complex form.

The results in Table 9 indicate that heat treatment before irradiation results in a greater loss of enzyme activity than equal amounts of energy given in the reverse order. It was also observed that after a rapid loss of activity with only mild heating, the rate of enzyme inactivation with increased heating time appeared to follow that of the unirradiated preparation.

The data in Fig. 8a,b,c, may be summarized as follows: 1) All rate curves showed a rapid initial drop in activity—approximating a discontinuous function with respect to heating time. 2) The rate of inactivation of lipoxidase, after the initial rapid decrease in activity, proceeded only as a function of heating temperature, independent of irradiation dose. 3) The lipoxidase preparation was inactivated to a greater extent under combined treatment when heated prior to irradiation. 4) The magnitude of the combined effects of heat and irradiation could not be predicted from the extent of the inactivation of the separate treatments.

Fig. 5 shows that a certain small fraction of the lipoxidase activity, present in the pea puree preparations, was rapidly destroyed upon heat treatment. This rapid initial loss of activity was attributed to the destruction of lipoxidase not bound as a stable complex with the pea solids.

The magnitude of the activity of the free enzyme could be estimated from these data by extrapolating to zero heating time the "first-order" rate-of-destruction curves obtained by heating the enzyme-pea puree solutions at any selected temperature for various lengths of time, and noting that the straight line did not pass through the "origin" (100% activity at zero heating time).

Similar analysis of the data for the combined treatment of the enzyme in the presence of pea solids gave the values in Table 9. At any one temperature, inactivation seemed to proceed at a rate independent of irradiation dose. The following interpretations are advanced to explain these data.

The treatment of food materials by ionizing radiation causes relatively little change in the physical and chemical state of the system. Irradiation of a solution of lipoxidase in the presence of pea solids, while not modifying the physical form of the system to any extent, seemed to cause a fraction of the active enzyme molecules to be destroyed immediately, and an additional fraction to be modified with respect to its sensitivity toward heat.

The portion of the activity destroyed immediately by ionizing energy may be termed the "apparent irradiation effect," and the fraction of the lipoxidase molecule changed by irradiation, with respect to heat resistance, may be termed the "latent irradiation effect." A summation of these may be called the "total effect" of irradiation.

Thus, it is postulated that, upon cessation of irradiation, a certain percentage of the lipoxidase originally present in the solution had been rendered inactive ($D_0 = 9 \times 10^4$ rad) and an unknown amount of the enzyme had been changed with respect to heat resistance (latent irradiation effect) and remained bound in an active form as an enzyme-pea solids complex. A third fraction existed unchanged, probably largely in complex form.

It is suggested that heat treatment of the irradiated lipoxidase preparation had the following effects: 1) the lipoxidase fraction modified by irradiation was rapidly and largely destroyed; 2) continued heat treatment destroyed the remaining bound stable enzyme at a rate equal to that observed for the unirradiated preparation.

The total irradiation effect of 0.6 and 2.0×10^6 rad may be estimated from the values given in Table 9 under the heading "Irradiate-Heat." It is found to be 38% at 0.6×10^6 rad and 50% at 2×10^6 rad. If the values obtained by irradiation in the absence of heating are then subtracted from the total-effect values, an estimate may be obtained of the latent irradiation effect. These values are about 25% at 0.6×10^6 rad and 22% at 2.0×10^6 rad. As the irradiation dose was increased from 0.6 to 2.0×10^6 rad the latent irradiation effect appeared to be decreased. This suggested that the protective effect of the pea puree-enzyme complex was decreasing and that at some greater irradiation level the results

of the combined effects of irradiation and heat, in that order, on lipoxidase in pea puree would first approach the values calculated for the separate effects of the two treatments, and then irradiation would actually sensitize the enzyme to heat.

The combined effects of heat and irradiation, in that order, may now be analyzed in light of the above postulates to see why this treatment was much more effective than either the reverse treatment or the individual treatments.

Heat treatment of lipoxidase in the presence of 20% pea solids at 67.5°C seemed to cause the rapid destruction of only a small amount of the free enzyme in the preparation, as shown in Fig. 5. Yet it may be suggested that the effect of the heat was quite profound on either the enzyme-pea solids complex or the complexing agent itself. An increase in the temperature of inactivation, from 67.5 to 72.5°C, showed an even greater effect on the complex and on the free enzyme in the absence of irradiation treatment.

It may be further suggested that heat treatment before irradiation either reversed or seemed to destroy the complexing ability of the pea solids toward free lipoxidase, and therefore greatly reduced the protective value of the pea puree against ionizing energy. Protective effect in this case is not meant with respect to radiation as radical scavenger but as an agent that allowed support and stabilization of the tertiary enzyme configuration against degradation through the various effects of ionizing energy on the enzyme in pea puree.

The data in Table 9 show that heat treatment before irradiation did greatly increase the effect of the ionizing energy. At 67.5°C the "zero heating time" intercept showed a loss of activity of only 5% (Fig. 5) and treatment of the preparation with 0.6×10^6 rad of gamma radiation alone gave a destruction of 12%. Together these treatments yielded a destruction of 50%. At 2×10^6 rad, sufficient to cause 28% destruction of the enzyme in the absence of heat, a combined treatment gave 77% inactivation.

An increase in treatment temperature had a similar effect at constant irradiation dose. In this case, 72.5°C allowed a destruction of 62% of the enzyme by an irradiation dose of 0.6×10^6 rad, whereas 67.5°C gave a 50% destruction. The reverse treatment order was shown to result in the same destruction at both 67.5 and 72.5°C.

These effects were observed by Leone *et al.* (1959) with crystalline ovalbumin treated with heat and ionizing energy.

The above may be considered from the viewpoint of the food processor contemplating the preservation of raw plant and animal materials by ionizing energy. Heat treatment of the food material, while not decreasing the "radical-scavenging" ability of the food, appears to reduce the ability of the food to form protective complexes with the enzymes in it, perhaps by altering the equilibrium of the "enzyme-food" complex.

Since the use of some heat appears highly desirable to obtain the fullest effect of the irradiation treatment (apparent and latent effects), the problem reduces itself to one of maximizing the destructive effects of any given combined treatment. Results with lipoxidase in pea puree suggest the use of heat before irradiation. Commerical practice dictates such a procedure since heat penetration is facilitated by small individual food pieces and irradiation treatment is most advantageous when carried out in conjunction with pre-packaged foods exposed in a fixed configuration. Perhaps the most effective treatment would be to heat and irradiate simultaneously. Proper dose rates and processing temperatures would depend on the product, type of irradiation source, and preservation objective, all of which need study.

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Stability of Spinach Catalase. I. Purification and Stability During Storage[a]

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SUMMARY

Some of the variables influencing the stability of purified and unpurified spinach catalase during storage were evaluated in model systems. A purified preparation of spinach catalase was obtained from spinach leaves by comminution and extraction with phosphate buffer and extraction with *n*-butanol. $(NH_4)_2SO_4$ precipitation, $(NH_4)_2SO_4$ fractionation, dialysis, and lyophilization. The preparation had a Kat.-f. of 3300 at pH 7.0. The stability of the enzyme in storage was influenced primarily by storage temperature, the pH of catalase solutions, and the activity of microorganisms. The enzyme concentration had a relatively minor effect on catalase stability. The stability of spinach catalase during storage was not influenced by the presence of NaCl, sucrose, starch, and denatured catalase in catalase solutions; the atmospheric pressure under which solutions were stored; or differences in freezing rates.

Although a number of studies of plant catalase have been reported, especially in the older literature as reviewed by Joslyn (1949), there has been surprisingly little fundamental interest in the storage stability, heat inactivation, and regeneration of this enzyme. These closely related aspects of stability should be better understood because of their pertinence to catalase research and to industrial applications of the enzyme.

Both Galston *et al.* (1951) and Mitsuda (1956) have shown that plant catalase is substantially less stable during storage than is catalase from other sources. Mitsuda (1956) and Mitsuda and Nakazawa (1954) reported that the stability of catalase extracted from rice-plant leaves was decreased by dilution. During 24-hr storage at room temperature, the enzyme was more stable in pH 7 phosphate buffer and in $NaHCO_3$ solution than in water or solutions of NaCl and other salts. Galston *et al.* (1951) found that purified spinach catalase was stable in the cold at a pH between 5.3 and 8.9, and was destroyed rapidly at lower pH values and slowly at higher pH values. According to Luck (1957), the pH effect is reversible above pH 4, probably reflecting the formation of the inactive secondary enzyme substrate complex.

Although research cited by Joslyn (1949) has shown other enzymes to be relatively stable at low temperatures, little attention has been given to the stability of plant catalase during prolonged frozen storage. Kiermeier (1947, 1949) observed losses in catalase activity in potato extracts and whole potatoes stored for as long as 2 wk at temperatures ranging from 2.5° to −15.4°C with the storage losses being greatest at the lowest temperatures. He also investigated the effects of repeated freezing and thawing and of differences in the rate of freezing.

These studies point out some of the variables likely to influence the storage stability of plant catalase. The present research was intended to evaluate the importance of these and other variables on the storage stability of purified and unpurified spinach catalase in model systems.

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EXPERIMENTAL

Purification of spinach catalase. The enzyme was partially purified using a modification of the techniques of Mitsuda and Yasumatsu (1955) and Galston *et al.* (1951).

All purification operations were carried out under refrigeration. Frozen spinach leaves (153 lb) were comminuted with a Hobart Food Cutter and suspended in 102 L of 0.01*M* pH 7.0 phosphate buffer. As described by Mitsuda and Yasumatsu (1955), the suspension was strained through cheesecloth, and the filtrate was extracted with 13 gal. *n*-butanol to liberate catalase from the suspended chloroplasts and bring it into aqueous solution. The aqueous phase was separated from the *n*-butanol phase, and precipitated solids were removed by continuous centrifugation on a Westphalia centrifuge. The supernatant was then 50% saturated with $(NH_4)_2SO_4$ to precipitate the catalase and other proteins. Catalase was brought into solution with 0.1*M* Na_2HPO_4 and was further purified by $(NH_4)_2SO_4$ fractionation.

Proteins precipitated at different levels of $(NH_4)_2SO_4$ saturation were separated by centrifugation for 20 min at 2200 rpm, washed with $(NH_4)_2SO_4$ solution, recentrifuged, and dissolved in 0.1*M* Na_2HPO_4. The fractionation scheme is summarized below.

Fractions were obtained at 0–13% saturation (a), and at 13–44% saturation (b). Fraction *a* consisted of soluble and insoluble protein; the latter was suspended in 0.1*M* Na_2HPO_4 (suspension *A*), and the former was further fractionated at 0–20% saturation (c), and at 20–50% saturation (d). Fractions *c* and *d* were dissolved in 0.1*M* Na_2HPO_4, yielding solutions *B* and *C*. Fraction *b* consisted of soluble and insoluble protein; the latter was suspended in 0.1*M* Na_2HPO_4 (suspension *D*), and the former was further fractionated at 0–14% saturation (e), and 14–24% saturation (f). Both fractions *e* and *f* consisted of soluble and insoluble protein, which were dissolved or suspended in 0.1*M* Na_2HPO_4, respectively yielding solutions *E* and *G* and suspensions *F* and *H*. Solutions *B*, *C*, *E*, and *G* were combined and concentrated by $(NH_4)_2SO_4$ precipitation, centrifugation, and resolution of the protein in a smaller volume of 0.1*M* Na_2HPO_4 to yield solution I. Suspensions *A*, *D*, *F*, and *H* were also combined and centrifuged to yield precipitate II. Precipitate II was partially solubilized by digestion with 0.1% trypsin. The soluble protein obtained from precipitate II was separated from solution by $(NH_4)_2SO_4$ precipitation and centrifugation, and was redissolved in fresh 0.1*M* Na_2HPO_4 and combined with solution I. The insoluble residue of precipitate II was discarded.

Attempts to increase the purity of the combined catalase solutions further by using starch-block electrophoresis (0.05*M* pH 7.0 phosphate buffer, 350 V, 18 hr at 2°C) and dioxane fractionation (at 35, 42, and 56% dioxane under refrigeration at 36–40°F) were not successful.

The soluble catalase preparation was dialyzed against distilled H_2O and then lyophilized. A yield of 1.34 g of dry enzyme preparation was obtained. The preparation had a Kat.-f. of 3300 at pH 7.0.

Determination of catalase activity. Manometric assays were employed with all impure and partially purified catalase samples (Maechly and Chance, 1954). The methods described by Goldstein (1949) and Burk and Hobby (1954) for free manometry with Barcroft-Warburg manometers were used. Assays were performed at 26°C. The catalase activity of samples was based on zero-order reaction-rate constants, expressed as μl of O_2 evolved per minute (μl/min).

Spectrophotometric assays based on the method of Beers and Sizer (1952) were used with solutions of purified catalase. A wavelength of 235 mμ and an H_2O_2 concentration of 0.0176*M* were employed. The catalase activity, based on the first-order reaction-rate constant k (sec^{-1}), was expressed as the Kat.-f. or as k per mg of protein in the sample (k/mg).

Storage studies. A series of studies was undertaken to determine the effects of storage temperature, storage time, pH, enzyme concentration, atmospheric pressure, bacterial activity, added NaCl, sucrose, starch, and denatured catalase, and freezing rates on the stability of spinach catalase. All preparative work was performed under refrigeration.

Catalase solutions containing 0.1, 0.2, and 0.4 mg of dry protein per ml were prepared by dissolving the enzyme preparation in 0.01*M* phosphate buffer at pH 5.5, 7.0, and 9.0. The solutions were dispensed into one- and 5-ml glass ampoules (Kimble), which were then heat-sealed either at atmospheric pressure or *in vacuo*. Ampoules were stored in dark rooms maintained at 86, 36–40, 0, and −40°F. The ampoules to be stored at the latter two temperatures were quick-frozen by immersion in dry ice–alcohol. Storage times varied from two hr to three months, depending on the storage temperature. After storage, duplicate ampoules were opened and the contents assayed with up to six replicates.

A series of 0.2 mg/ml–pH 5.5 catalase solutions were made up to contain 1.5% NaCl, 3.6% sucrose, 3.6% starch, and 0.2 mg/ml denatured enzyme preparation. These solutions were stored in air at 86°F for up to 5 hr.

The storage stability of catalase was also determined in spinach extracts simulating whole

spinach leaves. Extracts were prepared by blending 100 g of frozen spinach leaves with 50 g distilled H_2O in a Waring blender, centrifuging the homogenate 10 min at 2200 rpm, and filtering the supernatant through S & S no. 595 filter paper. The solids of spinach extracts were determined by evaporating samples to dryness on a steam bath. Extracts were stored in air at 86°F for as long as 36 hr. The pH of the extracts was determined at intervals during storage.

To determine the influence of bacterial activity on the stability of spinach catalase during storage, a 0.1 mg/ml–pH 7.0 solution of the purified enzyme preparation was sterilized by filtration through an HA Millipore filter and stored aseptically in parallel with an identical nonsterile catalase solution at 86°F. Both solutions were assayed after different storage intervals. Parallel experiments were also performed with spinach extracts containing 0 and 25 ppm of aureomycin (Lederle) stored at 86°F. Standard plate counts were made on spinach extracts and on solutions of the purified enzyme using tryptone glucose extract agar (Difco) and an incubation time of 24 hr at 86°F.

The effect of freezing rate on the stability of purified spinach catalase was determined by immersing ampoules of 0.2 mg/ml–pH 7.0 solutions in alcohol cooled to 0, −40, and −108°F. Frozen solutions were immediately thawed and assayed.

Rates of inactivation were determined by linear regression analysis when the inactivation process followed zero-order kinetics.

RESULTS AND DISCUSSION

Purification. The degree of purity of the catalase preparation used in this research represents a 50-fold increase in specific activity. Galston *et al.* (1951) reported that the maximal Kat.-f. of their highly purified crystalline spinach catalase was 23,600. Using a different procedure, Mitsuda (1956) obtained crystals of spinach catalase having a Kat.-f. of 15,300. If Galston's maximal value is assumed to represent the pure enzyme, then the preparation described herein contained about 14% catalase. Further purification would have been desirable but was not considered feasible within the confines of this research.

The 1.34 g of enzyme preparation represents a yield of about 10%. Losses in catalase activity undoubtedly resulted during the purification operations as a result of inhibition by acid impurities in the $(NH_4)_2SO_4$, foaming, trypsin digestion, and storage changes. Mitsuda and Nakazawa (1954) reported losses during dialysis, and Dounce and Schwalenberg (1950) observed catalase inactivation during lyophilization. It is possible that different configurations of the active catalase molecule were produced during purification by alteration of the native molecule.

Stability of spinach catalase during storage—effect of storage temperature. As might be expected, temperature was found to be the most important variable affecting the stability of spinach catalase during storage. Table 1 shows data representing the stability of 0.2 mg/ml–pH 7.0 solutions of purified spinach catalase stored in air and *in vacuo* at different temperatures.

It can be seen that the enzyme was inactivated rapidly at 86°F, underwent little or no change at 36–40° F, and was quite stable during frozen storage. This trend is consistent with results reported in the literature by Galston *et al.* (1951), Joslyn (1949), and Mitsuda (1956), and is probably a general characteristic of labile biological materials. Kiermeier's (1947, 1949) observations with catalase in frozen potatoes and potato extracts are difficult to reconcile

Table 1. Effect of storage temperature on the stability of 0.2 mg/ml–pH 7.0 solutions of purified spinach catalase in air and *in vacuo*.

Storage temperature (°F)	Storage time	Average specific activity (k/mg)	
		Air	Vacuum
86	0 hours	.152	.152
	6	.153	.150
	18	.102	.100
	24	.0843	.0701
	42	.0380	.0277
36–40	1 days	.160	.157
	3	.170	.148
	7	.160	.142
	14	.158	.147
0	7 days	.143	.155
	30	.152	.145
	60	.169	.158
	90	.153	.160
−40	7 days	.154	.146
	30	.164	.162
	60	.155	.173
	90	.166	.170

with the results of this research. They may reflect transient effects mentioned by Joslyn (1949), sampling or analytical variability, or some unusual effect of freezing on the catalase structure and its intracellular site in potatoes.

It is also apparent in Table 1 that the atmospheric pressure under which solutions of catalase were stored had no significant effect on the stability of the enzyme. This was also true under other conditions of storage discussed.

Effect of pH. The stability of purified spinach catalase during storage at a given temperature was found to be highly dependent on the pH of the enzyme solution. The effect of pH on the stability of 0.4 mg/ml solutions stored in air at 86°F is shown in Table 2.

Table 2. Effect of pH on the stability of 0.4 mg/ml solutions of purified spinach catalase stored in air at 86°F.

Storage time (hr)	Av. specific activity (k/mg)		
	pH 5.5	pH 7.0	pH 9.0
0	.0661	.162	.169
2	.352	.....	.....
4	.0345	.....	.....
6	.0360	.156	.132
18	.0137	.119	.104
24	.....	.0915	.0855
42	.....	.0504	.0571

The enzyme was inactivated at a much greater rate at pH 5.5 than at pH 7.0 or 9.0. The kinetics of inactivation were also found to vary with the pH of the catalase solution. At pH 7.0, zero-order kinetics were followed; at pH 9.0, the inactivation was first-order, and at pH 5.5, inactivation curves were concave on semilog paper. These trends were also observed during storage at 36–40°F. Even during frozen storage, pH 5.5 catalase solutions showed losses in activity during the first day.

Similar pH effects were reported by Galston *et al.* (1951). Since all catalase assays were performed at pH 7.0, the pH effects illustrated in Table 2 were obviously irreversible. The change in inactivation kinetics with pH suggests a complex mechanism of inactivation that probably involves the competitive inhibition in acid solutions described by Theorell (1951), the presence of several forms of catalase differing in stability, the protective effects discussed by Reiner (1959), and other factors. It is not likely that the extreme instability of pH 5.5 solutions represents denaturation, since rapid changes in activity occurred at all storage temperatures.

Effect of bacterial activity. Significant bacterial growth was found in solutions of purified spinach catalase at each pH and in aqueous spinach extracts stored at 86°F. This is not surprising since carbon, nitrogen, and other essential nutrients were present in the enzyme solutions and spinach extracts in amounts sufficient to permit the initial bacterial load to multiply.

The activity of these microorganisms had a profound effect on the stability of catalase in solution and in spinach extracts. The rates of inactivation of 0.1 mg/ml–pH 7.0 solutions of purified spinach catalase stored in air at 86°F were found to be 3.11%/hr for nonsterile solutions and 1.02%/hr for sterile solutions. Table 3 illustrates the effect of aureomycin on the stability of catalase in spinach extracts stored in air at 86°F.

In the extract containing aureomycin, catalase inactivation was greatly reduced compared to the extract containing no antibiotic. A portion of the effect of bacterial activity on the stability of spinach catalase may be due to acid production. The pH of the extract containing no aureomycin decreased from 6.62 to 5.90 in 24 hr, whereas the extract containing 25 ppm of aureomycin showed no change in pH during the same storage time.

The rates of inactivation of catalase in

Table 3. Stability of catalase in aqueous extracts of spinach containing 0 and 25 ppm aureomycin at 86°F.

Storage time (hr)	Average specific activity (manometric)	
	No aureomycin	25 ppm aureomycin
0	14,300	8,970
8	12,900	9,540
12	6,500	8,420
18	1,670	8,190
24	896	7,250
36		5,740

spinach extracts containing 25 ppm aureomycin and in sterile 0.1 mg/ml–pH 7.0 solutions at 86°F were similar, the former being 1.11%/hr and the latter 1.02%/hr. Since the enzyme is considered to be bound within the chloroplast structure in the former case and in colloidal suspension in the latter case, the comparable stability of the two preparations suggests that the mechanism of inactivation does not involve the intermolecular bonds between catalase and the chloroplast.

Effect of enzyme concentration. Under some conditions of storage, a relatively minor protective effect was found when the enzyme concentration was increased. This is illustrated in Table 4 by stability data obtained with pH 9.0 solutions of purified spinach catalase stored at 36–40°F.

Table 4. Effect of enzyme concentration on the stability of pH 9.0 solutions of purified spinach catalase stored in air at 36–40°F.

Storage time (days)	Average specific activity (k/mg) at:		
	0.1 mg/ml	0.2 mg/ml	0.4 mg/ml
0	.126	.164	.169
1	.154	.156	.186
3	.148	.142	.164
7	.124	.130	.153
14	.137	.126	.149
30	.109	.104	.124

Results were similar during the frozen storage of pH 7.0 and 9.0 solutions of purified spinach catalase.

Effects of other variables. The addition of 1.5% NaCl, 3.6% sucrose, 3.6% starch, and 0.2 mg/ml of denatured catalase to 0.2 mg/ml–pH 5.5 solutions of purified spinach catalase had no effect on the stability of the enzyme in air at 86°F. Mitsuda (1956) and Mitsuda and Nakazawa (1954) reported that NaCl had an adverse effect on the stability of rice-plant leaf catalase in this concentration range. The apparent conflict in results may be explained by differences in the enzyme source and in the pH of the preparation.

Freezing times varying from 1 min at −108°F to 4 min at 0°F did not influence the activity of a 0.2 mg/ml–pH 5.5 solution of purified spinach catalase. Kiermeier (1947, 1949) reported an effect with potato catalase over a broader range of freezing rates.

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Stability of Spinach Catalase. II. Inactivation by Heat[a]

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SUMMARY

The effects of certain variables on the thermal stability of spinach catalase were investigated with model systems. The rate of thermal inactivation of spinach catalase was accelerated as heating temperature was increased. Inactivation kinetics were not first-order with solutions of the purified enzyme between 50 and 60°C and with spinach extracts at 55°C, but became first-order with the latter preparation as the temperature was increased to 65°C. The presence of a heat-labile catalase inhibitor is postulated. Catalase was more thermostable in spinch extracts than in solutions of the purified enzyme. Purified spinach catalase at 55°C was more thermostable in solutions at pH 5.5 and 7.0 than at pH 9.0. The thermostability of purified spinach catalase was not influenced by the enzyme concentration or by the presence of 1.5% NaCl, 3.6% sucrose, or 3.6% starch in pH 7.0 solution at 55°C.

The thermal lability of catalase and many other enzymes has been well established. However, basic studies of heat inactivation have been confined to catalase from animal and microbiological sources. Values of ΔH^* and ΔS^* for inactivation have been determined for crystalline beef-liver catalase by Frazer and Kaplan (1955), Guild and van Tubergen (1957), and Sizer (1944), for intracellular yeast catalase by Frazer and Kaplan (1955), and for crystalline horse erythrocyte catalase by Deutsch (1951).

According to Sizer (1944), Frazer and Kaplan (1955), and Guild and van Tubergen (1957), the thermal inactivation of beef-liver catalase is a first-order reaction. However, Deutsch (1951) reported that the inactivation of crystalline horse erythrocyte catalase is not first-order, the rate decreasing rapidly with time. He suggested that a number of species of catalase were present in his preparation, each differing in thermal lability. Frazer and Kaplan (1955) observed, during the heat inactivation of intracellular yeast catalase, an initial lag phase that they attributed to the weak linkages holding catalase to the intracellular interfaces.

Frazer and Kaplan's results indicated that the thermal lability of yeast catalase is not greatly influenced by pH. Deutsch (1951) reported that crystalline horse erythrocyte catalase is most stable to heat near the isoelectric point of the enzyme. Kiermeier and Köberlein (1957) found that mono- and disaccharides increased the heat stability of crystalline catalase.

For the most part, investigations of the thermal lability of plant catalase have dealt with semiquantitative determinations of blanching times and temperatures. This work was discussed in detail by Joslyn (1949). In general, the data are limited in scope and give little insight into the kinetics and mechanism of catalase inactivation by heat. In the present study, certain fundamental characteristics of the thermal inactivation of spinach catalase were examined in model systems with quantitative techniques.

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[c] This paper is based on research carried out by G. M. Sapers for the Ph.D. degree in Food Technology at Massachusetts Institute of Technology.

EXPERIMENTAL

Source of enzyme. Determinations of the heat lability of spinach catalase were carried out in solutions of the purified enzyme preparation and aqueous spinach extracts as described earlier (Sapers *et al.*, 1962).

Equipment and heating procedures. Solutions of the purified enzyme preparation in 0.01*M* phosphate buffer were heated in 18 × 150-mm test tubes and also in sealed 1.5–2.0 × 100-mm glass melting-point capillary tubes having an inner diameter of 1.2–1.4 mm; aqueous spinach extracts were heated in capillary tubes. The end of each capillary was fused to a short supporting section of capillary tube bent in the shape of a cane. The capillary tube filling, sealing, and heating procedures of Stern and Proctor (1954), as modified by Licciardello (1960) and Farkas (1960), were used. With this technique it was possible to heat 0.075-ml samples under precisely controlled conditions with minimal heating and cooling lags.

The capillary-tube technique was not practical when the catalase activity was reduced to a low level by exposure to heat, since the contents of a great many tubes would have to be pooled to provide sufficient solution for assaying. Consequently, samples to be given extended exposures to heat were heated in test tubes. In order that heat penetration lags be minimized, a small volume of concentrated enzyme solution (1–2 ml) was added rapidly by serological pipette to 15–20 ml of preheated buffer in the test tube and immediately mixed in with the tip of the pipette. After each heating interval, portions of the solution were transferred rapidly by serological pipettes from the heating test tube to chilled test tubes immersed in an ice bath.

Methods of assay. Preliminary experiments demonstrated that the contents of capillary tubes could not be released by crushing in buffer, as is usually done, following the method of Stern and Proctor (1954), since a large proportion of the catalase activity was lost by inactivation on the surface of the ground glass. As an alternative method, the capillary tubes were broken at each end, allowing the contents to drain into test tubes chilled by immersion in an ice bath. The empty capillaries were then flushed with a measured volume of buffer injected with a tuberculin syringe. The contents of as many as 60 capillary tubes, heated under identical conditions, were pooled by this procedure to allow for 6 replicate assays at all activity levels. Recovery of catalase activity was found to be quantitative.

After pooling and appropriate dilution, solutions of purified spinach catalase were assayed by a spectrophotometric method described earlier (Sapers *et al.*, 1962). Aqueous spinach extracts, after pooling and appropriate dilution, were assayed by the manometric method described in an earlier paper.

Presentation of data and correction for heat-penetration lags. The experimental data were treated graphically by plotting average specific activity against heating time on semilog paper.

Significant heat penetration lags occurred in catalase solutions heated in test tubes. The extent of catalase inactivation was greater in solutions heated in capillary tubes than in test tubes for the same heating time and temperature. It was observed that inactivation curves based on the test-tube technique showed an increasing positive deviation from the capillary-tube inactivation curves until the test-tube come-up time had been exceeded. At that point the curves became parallel. Between 55 and 65°C, come-up times varied from 2 to 3 min.

Correction was made for the effect of the heat-penetration lag by graphically subtracting the constant difference between the two curves from all points obtained by the test-tube technique at heating times greater than the come-up time. The capillary-tube technique was used for all heating times less than and including the come-up time.

Experimental design. The rate of heat inactivation of spinach catalase in solutions of the purified enzyme was determined under the following conditions:

Enzyme concentration: 0.2, 0.4, 1.0 mg enzyme preparation per ml of 0.01*M* pH 7.0 phosphate buffer, heated at 55°C.

pH: 1.0 mg/ml buffer solutions at pH 5.5, 7.0, and 9.0 heated at 55°C.

Heating temperature: 1.0 mg/ml-pH 7.0 solutions heated at 50, 55, and 60°C.

Added solids: 1.0 mg/ml-pH 7.0 solutions containing 1.5% NaCl, 3.6% sucrose, and 3.6% starch, heated at 55°C.

In addition, aqueous spinach extracts were heated at 55, 60, and 65°C.

RESULTS AND DISCUSSION

Effects of heating temperature. Fig. 1 shows the effect of heating temperature on the rate of inactivation of spinach catalase in solutions of the purified enzyme. It can be seen that heating temperature had a very important effect on the stability of spinach catalase. The time required to inactivate 50% of the enzyme was estimated to be 4 min at 50°C, 30 sec at 55°C, and 10 sec at 60°C. It is also evident that the inactivation rate was not first-order. The enzyme was inactivated very rapidly initially,

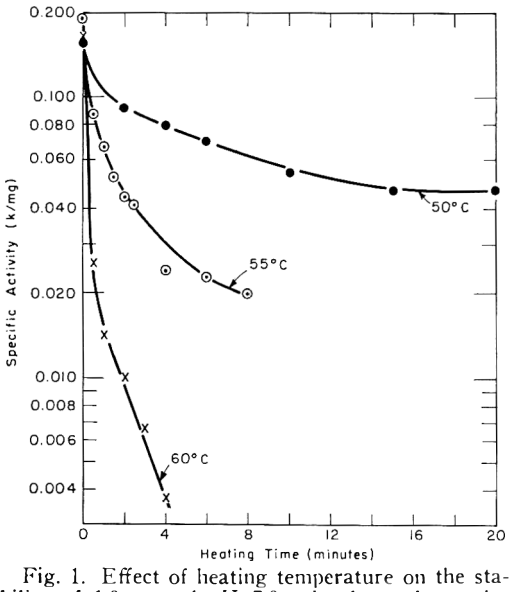

Fig. 1. Effect of heating temperature on the stability of 1.0 mg/ml–pH 7.0 spinach catalase solutions.

but the rate progressively decreased as the heating time was increased.

As can be seen in Fig. 2, heating temperature had a similar effect on the stability of catalase in spinach extracts. However, the enzyme showed a greater degree of stability in spinach extracts. The time required to reduce the activity by 50% was estimated to be 8–13 min at 55°C, 2.5 min at 60°C, and 13–15 sec at 65°C. Inactivation curves were nonlinear at 55°C but became first-order as the temperature was increased to 65°C.

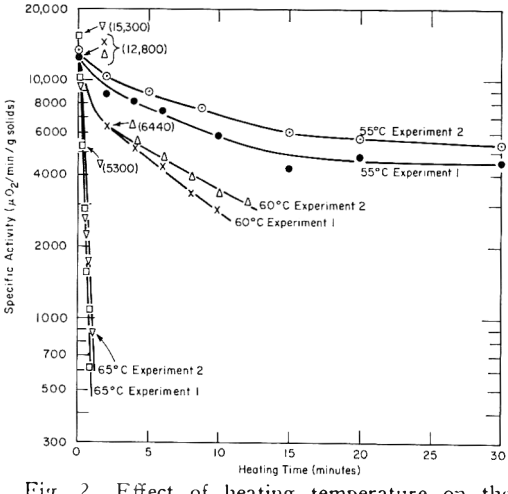

Fig. 2. Effect of heating temperature on the stability of catalase in spinach extracts.

Activation enthalpies and entropies, based on the linear portions of the curves in Fig. 2, were calculated between 55 and 60°C and between 60 and 65°C. These values, given in Table 1, are only approximate since the relation between the logarithm of the first-order rate constant and reciprocal absolute temperature was not determined for more than three temperatures.

Table 1. Thermodynamic constants for the inactivation of catalase in spinach extracts.

Temperature range (°C)	Calculation temperature (°C)	ΔH^* (kcal/mole)	ΔS^* (kcal/mole °K)
55–60	60	25.5	5
60–65	60	60.9	111

The nonlinear inactivation curves obtained with purified spinach catalase may indicate the presence of a number of species of the catalase molecule formed by alteration during purification from the native molecule, each of which differed in heat lability.

The shape of inactivation curves may also be explained by assuming the presence of a heat-labile catalase inhibitor. Initially, catalase would be inactivated not only by heat but also by the temperature-accelerated action of the inhibitor. If the inhibitor were heat-inactivated more rapidly than catalase, its inactivating effect would disappear with time, and the slope of the inactivation curves would decrease, eventually becoming constant when heat denaturation alone caused the loss in activity. This trend can be seen in Figs. 1 and 2.

The transition of inactivation curves obtained with spinach extracts from less than first-order to first-order with increasing temperature is also indicative of a heat-labile inhibitor mechanism. It is necessary to assume that the inhibitor, if present, was in close proximity to catalase as it exists in the chloroplast structure of the plant, and that inactivation of the inhibitor has a higher Q_{10} than inactivation of spinach catalase.

Final evidence supporting the inhibitor hypothesis can be seen in Table 1. The

values of ΔH^* and ΔS^* between 55 and 60°C are consistent with chemical inhibition of catalase, and values between 60 and 65°C are typical of catalase denaturation as reported by Deutsch (1951), Frazer and Kaplan (1955), and Guild and van Tubergen (1957).

It is unlikely that the nonlinear shape of inactivation curves resulted from the presence of several species of native spinach catalase in the purified preparation and in spinach extracts. If several species differing in lability to heat had been present, inactivation curves obtained with spinach extracts would probably be nonlinear at all temperatures.

It is also unlikely that a protective effect such as that described by Reiner (1959) was responsible for the nonlinear inactivation curves, since the kinetics predicted by his theoretical analysis were not observed in these experiments.

Effect of pH. The pH of solutions of purified spinach catalase was found to have an important effect on the thermal stability of the enzyme. This is indicated in Fig. 3.

The stability of 1.0-mg/ml solutions of the purified enzyme at 55°C was far less at pH 9.0 than at pH 5.5 or 7.0 Inactivation curves at the latter two pH values were parallel, indicating similar thermal lability. As discussed in an earlier publication (1961), the displacement of these curves reflected the initial instability of pH 5.5 catalase solutions during storage.

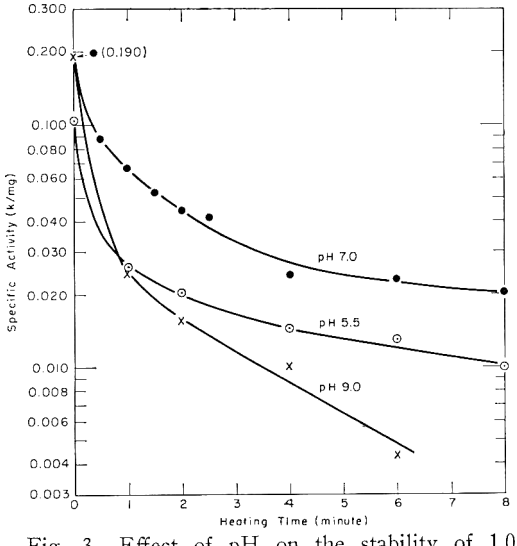

Fig. 3. Effect of pH on the stability of 1.0 mg/ml spinach catalase solutions at 55°C.

These results support the general observation made by Neurath *et al.* (1944) that the pH of maximal thermal stability for enzymes lies between the isoelectric point, pH 5.7 for beef-liver catalase according to Sumner and Dounce (1955), and neutrality. Similar results were reported by Deutsch (1951) and Frazer and Kaplan (1955), respectively for horse erythrocyte and intracellular yeast catalase.

Effects of other variables. The thermal stability of purified spinach catalase at 55°C was not influenced by the enzyme concentration between 0.2 and 1.0 mg/ml in pH 7.0 buffer. This provides further evidence against a protective mechanism such as described by Reiner (1959).

The presence of 1.5% NaCl, 3.6% sucrose, and 3.6% starch in 1.0 mg/ml-pH 7.0 solutions of purified spinach catalase had no effect on the stability of the enzyme at 55°C. The large protective effect of sucrose reported by Kiermeyer and Köberlein (1957) apparently does not apply under the experimental conditions employed in this research. It also appears that the greater thermal stability of catalase in spinach extracts than in solutions of the purified enzyme is due to the specific internal structure of the chloroplast rather than to nonspecific protective effects of soluble solids.

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Stability of Spinach Catalase. III. Regeneration[a]

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SUMMARY

The regeneration of spinach catalase was demonstrated during storage after heat inactivation. The occurrence of regeneration was influenced by the enzyme source, pH, heating conditions, and storage conditions. The change in catalase activity after heating was considered to represent the balance between enzyme regeneration and enzyme inactivation during storage.

Evidence for the regeneration of catalase after heat inactivation has been scanty, largely qualitative, and contradictory. One of the earliest reports of catalase regeneration was made by Diehl *et al.* (1936), who noted a return of activity in Alderman peas ½ hr after scalding and cooling. According to Joslyn (1949), catalase regeneration during the frozen storage of vegetables was observed when qualitative tests for catalase activity were used. More recently, data suggesting catalase regeneration have been reported in asparagus 15–30 min after blanching at 180°F by Paul *et al.* (1952), in dried spinach and potatoes after blanching by Virmani and Tandon (1950), and in peas stored at −10°F for as long as 13 months after a 20-sec blanch at 192–208°F by Dietrich *et al.* (1955). Regeneration has also been observed with dry crystalline beef-liver catalase as 60°C by Kiermeyer and Köberlein (1955), and with crystalline horse erythrocyte catalase at temperatures below 60°C by Deutsch (1951).

On the other hand, Sizer (1944) found no evidence of regeneration after crystalline beef-liver catalase was heat-inactivated. Lopez *et al.* (1954) failed to observe catalase regeneration in five varieties of southern peas blanched for 1 and 2 min in boiling water and stored at 0°F for as long as 29 months.

Little attention has been given to the systematic determination of heating and storage conditions under which plant catalase regenerates or fails to regenerate. In this investigation, some of these conditions were examined in an attempt to determine the nature of regeneration.

EXPERIMENTAL

Source of enzyme and methods of assay. The preparation and assay of samples of spinach catalase were described in detail earlier (Sapers *et al.*, 1962a, b). Regeneration studies were carried out on solutions of purified spinach catalase and on aqueous spinach extracts. A spectrophotometric assay procedure was used to determine catalase activity in the former samples, and a manometric method in the latter. All assays were performed with five or six replicates.

Heat inactivation. The capillary-tube heat-inactivation technique employed in earlier studies of spinach catalase stability (Sapers *et al.*, 1962b) was used in this investigation of catalase regeneration. Solutions containing 1.0 mg of enzyme preparation per ml of 0.01*M* phosphate buffer at pH 5.5, 7.0, and 9.0 and aqueous spinach extracts were heated under conditions sufficient to inactivate 70–80% of the catalase. Temperatures and times used to accomplish this degree of inactivation are summarized in Table 1. Samples were assayed for catalase activity before and immediately after heating.

Storage. After heating, capillary tubes containing catalase samples were immediately placed in storage at 86 and 36–40°F. Samples were taken

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[c] This paper is based on research carried out by G. M. Sapers for a Ph.D. degree in Food Technology at Massachusetts Institute of Technology.

Table 1. Heating conditions for the determination of spinach catalase regeneration.

Enzyme preparation	pH	Heating temperature (°C)	Heating time
Catalase solution	5.5	60	10 sec
Catalase solution	7.0	62	10 sec
Catalase solution	7.0	50	20 min
Catalase solution	9.0	57	10 sec
Spinach extract	....	67	10 sec

for assay after 2, 6, and 24 hr at 86°F, and after 1 and 7 days at 36–40°F.

RESULTS AND DISCUSSION

As can be seen in Table 2, these experiments provided considerable evidence of spinach catalase regeneration. There is little doubt that significant regeneration occurred with purified spinach catalase at pH 7.0 and pH 9.0, but not at pH 5.5. Statistically significant regeneration was not observed with catalase in spinach extracts, although an increase in catalase activity was noted during storage at 86°F.

These results illustrate several important characteristics of enzyme regeneration. The process is usually dependent on pH. According to Joly (1955) and Lumry and Eyring (1954), ionic bonds are involved in both denaturation and aggregation reactions. The likelihood of regeneration will depend on the extent to which the ionic bonds of the native protein may be restored at different pH's during storage.

It can also be seen that regeneration at pH 7.0 was substantially greater when samples were heated at 62°C than at 50°C. Fram (1957) and Zoueil and Esselen (1959) have shown that reversible denaturation is generally favored if HTST heat-

Table 2. Regeneration of spinach catalase.

Enzyme preparation	Heating conditions	Storage temperature (°F)	Storage time (hr)	Percent regeneration
pH 7.0 soln	10 sec at 62°C	86	2	29.0***
			6	17.3***
			24	4.65
		36–40	24	9.30***
			168	7.60**
pH 7.0 soln	20 min at 50°C	86	2	10.8***
			6	4.50
			24	0
		36–40	24	0
			168	0
pH 9.0 soln	10 sec at 57°C	86	2	16.7***
			6	0
			24	0
		36–40	24	14.0***
			168	0
pH 5.5 soln	10 sec at 60°C	86	2	0
			6	0
			24	0
		36–40	24	0
			168	0
Spinach extract	10 sec at 67°C	86	2	4.85
			6	16.3
Spinach extract	10 sec at 67°C	86	24	0
		36–40	24	0
			168	0.76

** Regeneration significant at 0.01 level.
*** Regeneration significant at 0.001 level.

ing conditions are employed. According to Lumry and Eyring (1954), with rapid heating, the relatively slow and irreversible aggregation reactions do not become limiting. This fact may explain the different results obtained by Lopez *et al.* (1954) and Dietrich *et al.* (1955) with pea catalase.

The results also demonstrate that catalase regeneration is an incomplete process, the residual activity increasing by only 29% at the most. Completely reversible denaturations are not common, and, in the opinion of Neurath *et al.* (1944), unlikely to occur.

Regeneration is usually dependent on the conditions of storage. Fram (1957) found alkaline phosphatase regeneration to be accelerated by temperature and to increase with time. However, while the results showed more regeneration at 86°F than at 36–40°F, there was a decrease in residual activity during extended storage. It was reported elsewhere that unheated spinach catalase solutions are unstable during storage. Thus, the process of regeneration is in competition with a simultaneous inactivation process, and changes in residual activity during storage after heating reflect the balance of the two effects. This may account not only for the disappearance of regeneration with time at pH 7.0 and pH 9.0 but for the complete absence of regeneration at pH 5.5 as well. At the latter pH, spinach catalase was found to be highly unstable during storage.

Heating appeared to stabilize catalase solutions during storage, even when no overt regeneration was observed. Table 3 illustrates the effect of heating on the retention of activity during storage. The increased storage stability of heated catalase solutions may be due to regeneration or to the destruction of bacteria or enzymes that would otherwise inactivate spinach catalase.

The absence of significant catalase regeneration in aqueous spinach extracts cannot be explained by competing inactivation reactions during storage, since the enzyme in spinach was found to be relatively stable under the conditions of this experiment. It is possible that denatured catalase in spinach extracts is constrained within the chloroplast structure in such a way as to prelude regeneration.

Table 3. Effect of heating on the retention of activity of purified spinach catalase during storage at 86°F.

pH	Storage time (hr)	% retention of catalase activity		
			Heated	Unheated [a]
5.5	2		78.9	53.2–70.4
	6		60.0	27.1–54.5
	18		.....	11.8–35.7
	24		36.8	.....
7.0	6	HTST	Slow Heating	
		118	104	86.2–101
	24	105	78.6	38.8–78.9
9.0	6		93.0	73.5–87.4
	24		73.3	50.5–64.5

[a] Based on data reported by Sapers *et al.* (1962b).

It is also possible that regeneration did occur, but at a level too low to be demonstrated statistically because of the relatively high variability of the manometric method of assay.

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Pectin Methylesterase in Snap Beans[a,b]

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(Manuscript received January 7, 1962)

SUMMARY

Snap bean pectin methylesterase had a pH optimum of 8.2. Its activity was enhanced by the presence of salts such as sodium chloride and calcium chloride. Low pH conditions increased the susceptibility of the enzyme to heat inactivation. There were indications that conditions were present in the intact pod which protected the enzyme from heat denaturation. The enzyme had a Q_{10} of 1.4 over a wide temperature range. It was not regenerated on frozen storage.

Pectin methylesterase is found in many plants (Lineweaver and Jensen, 1951) and is capable of catalyzing the deesterification of methyl galacturonide units of pectin to yield galacturonic acid units and free methyl alcohol. The enzyme is believed to be relatively inactive in most intact plant tissues, but when tissues are ground or macerated the esterase rapidly converts pectins to pectic acids. The enzyme has been of interest in foods because of the part that pectic substances play in influencing the structure and character of foods of plant origin. Pectin methylesterase has been studied in connection with cloud in orange juice (Guyer *et al.*, 1956), the texture of tomato products (Kertesz, 1938) and the texture of canned cauliflower (Hoogzand and Doesburg, 1961). The action of the enzyme results in a decreased solubility of the pectic substances, particularly in the presence of calcium salts, and an increased firmness of tissues.

Texture or firmness is an important factor in evaluating the quality of processed snap beans. Reports of Downey (1959), Sistrunk (1959, 1960), and McConnell (1956), and from our laboratories (Van Buren *et al.*, 1960a,b), have shown that the firmness of canned beans was greatly influenced by blanching conditions. Differences in firmness were related to the types of pectic substances present: the firmer beans had more pectic acid or pectate than the softer beans. Conditions under which firming occurred indicated that an enzyme was involved since changes in firmness were stopped when beans were heated to high temperatures. The nature of the chemical change that took place indicated that the enzyme was pectin methylesterase. The role of moderate blanching temperatures in initiating the action of the enzyme is a subject of continuing investigation. Such a phenomenon, however, has also been observed with ascorbic acid oxidase, and the action of mild heat in starting the enzymatic reaction duplicates in some ways the effect of macerating the plant tissues.

Pectin methylesterase has been reported in bean pods by Sistrunk (1959). The amount present depended on variety. The present paper describes some of the properties of the enzyme, its survival in frozen beans, and the effects of temperature and pH on its activity and denaturation.

METHODS

The enzyme was studied in Tendergreen snap bean pods either fresh, frozen, or as an acetone powder preparation. Enzyme activity was measured in the presence of 0.5% pectin by maintaining pH at the desired level through continuous titration with 0.05*N* sodium hydroxide (Kertesz, 1937) (Van Buren *et al.*, 1960b). The enzyme in

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[b] Journal Paper 1287, New York State Agricultural Experiment Station, Cornell University, Geneva, New York.

fresh pods hydrolyzed 27.9 microequivalents (μeq) of ester per gram dry weight per minute at pH 6.5 and 30°C. The acetone powder split 34.7 μeq of ester per minute per gram dry weight under the same conditions.

RESULTS

The activity of bean pectin methylesterase was influenced by pH and salt. Fig. 1 shows a pH

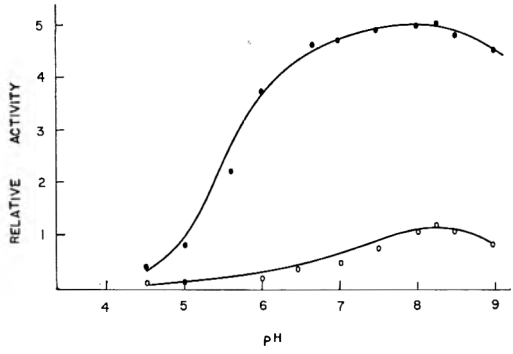

Fig. 1. The effect of pH on the activity of snap bean pectin methylesterase, acetone powder suspension. ○——○ no salt in assay medium. ●——● 0.15M sodium chloride in assay medium.

optimum for the enzyme of around 8.2, somewhat higher than the average pH of 6.5 normally found in bean pods. In the presence of sodium chloride the activity of the enzyme was increased and the range of pH conditions conducive to high activity was broadened. The effectiveness of sodium chloride depended on its concentration (Fig. 2). As the concentration was increased to about 0.2M the activity of the enzyme also increased, but higher concentrations depressed enzyme activity. Calcium chloride also enhanced the rate of enzymatic deesterification and was

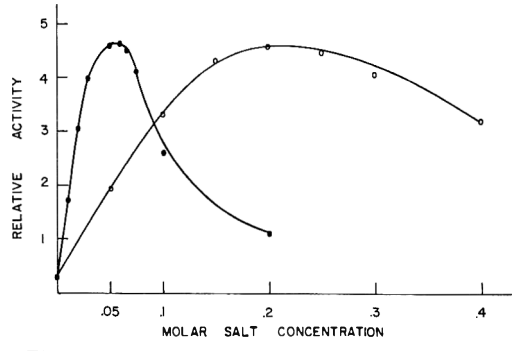

Fig. 2. Effect of salt concentration on the activity of snap bean pectin methylesterase, acetone powder suspension, pH 6.5. ○——○ sodium chloride. ●——● calcium chloride.

effective at lower concentration than sodium chloride. Other experiments using combinations of the two salts indicated that their effects were additive both in enhancing enzyme activity at suboptimal concentrations and in inhibiting the enzyme at high salt concentration. Mixtures of the salts did not result in greater activity than could be obtained with optimal concentrations of either salt alone. This suggests that both salts affected the enzyme by a similar mechanism. Other salts that enhanced the activity of the enzyme were ammonium chloride, potassium chloride, and magnesium chloride. In the intact bean pod the activity of the enzyme is probably influenced by the salts naturally present in the pod. The activity indicated in our assays in the absence of salt may be somewhat lower than what could be expected in the bean pod, because the pod components were diluted 15 or more times in our assay medium.

The enzyme as it occurred in the pod was more resistant toward heat denaturation at pH 6.5 than the enzyme in the form of a suspension of an acetone powder (Fig. 3). This suggests that there

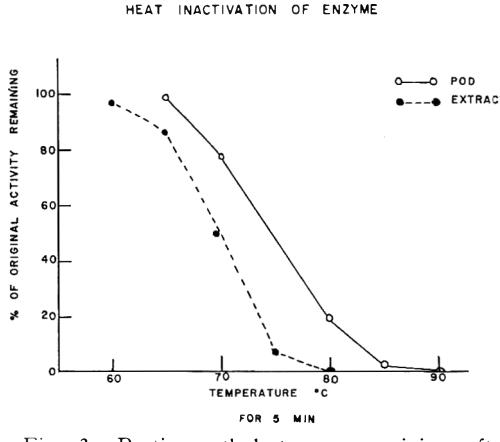

Fig. 3. Pectin methylesterase remaining after heating 5 min at various temperatures in 0.01M sodium phosphate buffer, pH 6.5. ○——○ pods. ●——● acetone powder suspension.

may be factors or conditions in the pod that protect the enzyme from inactivation. If such factors can be determined it may be possible to decrease the rate of enzyme inactivation during heating by altering some pod characteristics.

The interaction of pH, temperature, and time (Table 1) shows that the enzyme was less stable under conditions of low pH. This indicates that variations of pH conditions in the pods may influence not only the activity of the enzyme but also the degree to which the enzyme can survive a heat treatment or blanching procedure.

Table 1. Residual pectin methylesterase after heat inactivation at various temperatures and pH.[a]

pH at which heating took place	Percent of enzyme active after heating at:								
	65°C			70°C			75°C		
	2 min.	5 min.	15 min	2 min.	5 min.	15 min.	2 min.	5 min.	15 min.
4.5	10	3	0	3	0	0	0	0	0
5.0	46	21	1	31	6	0	2	0	0
5.5	85	72	59	42	27	16	15	2	0
6.0	87	69	61	51	35	29	12	4	1
6.5	95	86	83	59	49	35	18	7	5
7.0	93	92	87	60	47	38	15	10	6
7.5	94	81	70	47	34	22	20	8	5

[a] Enzyme heated as a suspension of an acetone powder in 0.01*M* phosphate buffer at the indicated pH.

In the temperature range of 20–50°C the enzyme had a Q_{10} value of about 1.4. When corrections were made for denaturation above this temperature, the Q_{10} stayed near 1.4 up to 75°C. Beyond 75°C it was impossible to extrapolate with any confidence.

The survival of the enzyme in frozen beans is shown in Table 2. To test for possible regeneration of the enzyme, the beans were blanched at different temperatures before freezing. The results show that the enzyme was not greatly affected by freezing and storage, and also, perhaps more important, the enzyme that had been destroyed by blanching was not regenerated on frozen storage.

Table 2. Retention of pectin methylesterase in frozen beans.

Blanch temperature [a] (°C)	Relative enzyme content	
	Before freezing	After 1-year storage at −18°C
No blanch	100	91
77	52	47
82	16	12
88	0	0
93	0	0
99	0	0

[a] Five-minute blanch.

DISCUSSION

Pectin methylesterase of green beans is qualitatively similar to the enzyme found in other plants, such as tobacco (Holden, 1946) and alfalfa (Lineweaver and Ballou, 1945) with regard to the influence of cations and pH on its activity. The level of activity is, however, about one-tenth that found in those other sources. Even so, there was sufficient enzyme in the beans to catalyze the demethylation of significant amounts of pectin. The activity of the enzyme in the presence of salts was quite sensitive to pH changes in the region around pH 6.5. Earlier work (Van Buren *et al.*, 1960b) has shown that the pH of the intact pods decreased several tenths of a unit as pectate was formed. Newly formed pectate would also be expected to bind calcium ions. It appears that the action of the enzyme in intact pods would tend to create conditions decreasing its activity.

There are many indications that the firmness of processed snap beans is related to the amount of pectic acid or pectate that is present to serve as an intercellular cement. In general, the amount of pectate present in fresh beans is not enough to prevent the processed bean from having a soft, sloughing character. Pectate levels can be increased by using processing conditions that allow the pectin methylesterase to act on the pectin.

The enzyme, as studied in Tendergreen beans, was inactivated rapidly at high blanching temperatures. Since, for other reasons, a high blanching temperature is often desirable, it might be useful to increase the activity of the enzyme in the pods by raising the pH on the pods, increasing the amount of enzyme, increasing its resistance of heat inactivation, or increasing the cation concentration. Such conditions might be achieved through breeding or modification of cultural and processing procedures.

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Relation Between Visual Color Differences and Tristimulus Color Readings for Pureed Carrots, Spinach, and Pears

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SUMMARY

A range of colors for spinach, carrots, and pears was produced by blending samples of different colors and diluting with water. The samples were examined by a visual panel under a MacBeth Examolite to determine the "just noticeable color difference" (JND) and were then measured with a Colormaster differential colorimeter and a Color-Eye colorimeter. The JND figures were 1–2.5 delta-E units calculated by the Adams chromatic value equation. Repeatability figures on the instruments above were respectively ½ and ¼ of the JND values for spinach and pears. For carrots, the repeatability and JND figures were of the same order. Repeatability figures for 4 other colorimeters were lower or approximately equal to the JND figures, indicating that several of the colorimeters could be used to determine color differences as well as an average visual panel.

INTRODUCTION

Methods of expressing the difference in color between 2 food samples in terms of a single number have been the subject of considerable research in recent years. Historically, the color difference has been judged visually, but the recent introduction of a number of different types of reflection colorimeters has simplified the problem. Current interest in a single number to represent a color difference has involved 2 main areas. The first is color grading where the color is an index of economic worth, as, for example, with tomato products, where the redder color is associated with better flavor. The second is production control, as illustrated by coffee or peanut butter, where the manufacturer desires to maintain a uniform color.

Control of color in manufacturing processes infers that a set of tolerances be established for each product. To establish such control limits it is desirable that the lower limits of sensitivity of the human eye for a given product be known. It makes little sense to attempt to control a process beyond the point where the eye could detect a difference under normal viewing conditions.

The measurement of color involves a 3-dimensional concept, and most color-difference formulae depend on a mathematical weighting to reduce 3 coordinates to one numerical value. This concept has been well developed for fields other than food (Judd, 1952). Perhaps the best known examples in the food field are tomato products. Younkin (1950) developed weightings for the visual differences in hue, chroma, and lightness without attempting to combine them. Others have developed a 2-dimensional concept (Smith and Huggins, 1952) and a 3-dimensional method (Yeatman *et al.*, 1960) of expressing colors of tomato products by a single figure. Several other formulas have been developed for tomatoes (Kramer, 1950), apples (Francis, 1952), flour (Croes, 1961), sugar (Deitz, 1956), grapefruit (Lime *et al.*, 1956), oils (Pohle and Tierney, 1957), beer (Stone, 1954), and many other foodstuffs. The delta-E concept of expressing color differences as a single figure has been used for thickening agents (Kiratsous

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The research reported was taken from a Ph.D. thesis by the senior author, whose present address is University of Philippines, Quezon City, Philippines.

et al, 1962) and high-temperature short-time processing studies on spinach (Tan and Francis, 1962), but it has not received wide acceptance in the food field.

This research reports an attempt to define the "just noticeable color difference" (JND) in terms of a 3-dimensional color-difference formula for pureed carrots, spinach, and pears, and its relationship to some tristimulus instruments that might be used to measure it.

MATERIALS AND METHODS

The pureed carrots, spinach, and pears used in this work were commercial baby-food samples obtained through the courtesy of the Gerber Co., Freemont, Michigan; H. J. Heinz & Co., Pittsburgh, Pennsylvania; Beech-Nut Baby Foods Div., Canajoharie, N. Y., and Libby, McNeill and Libby, Blue Island, Illinois.

The visual examinations were performed on unlabeled 200 × 309 5-oz baby-food jars on a stainless-steel bench under a MacBeth Examolite (MacBeth Daylighting Corp., Newburgh, N. Y.) mounted 4 ft above the bench. It was realized that the illumination and surroundings were very important in visual judgments, and that this type of viewing procedure was not likely to be found in a situation where a purchaser was choosing the product. The viewing situation was reproducible, however, and more or less definable, even though it was probably more critical than the usual consumer situation.

The instruments used were:

1) Color-Eye Colorimeter, Model D (Instrument Development Laboratories, Attleboro, Massachusetts). The instrument was operated in the normal manner, with a white Vitrolite tile over one port and a rectangular cell containing the sample over the other port. For carrots and spinach, a cell with a 2-cm depth of sample was used whereas a 4-cm cell was used for pears.

2) Colormaster Differential Colorimeter (Engineering and Equipment Corp., Hatboro, Pennsylvania). The instrument was operated with a white Vitrolite tile over the reference port for carrots and pears. For spinach, a 10× multiplier was used with a dark-gray tile ($G = 5.52$, $R = 5.49$, $B = 6.22$) for standardization. Circular cuvettes of 2¼ in. diam. and 1½ in. depth with optical glass bottoms were used with all instruments except the Color-Eye.

3) Gardner Color and Color-Difference Meter, Manual Model (Gardner Laboratory Inc., Bethesda 14, Maryland). The instrument was set up with large-area illumination and a 2-in.-diam. circular opening. Tiles with respective Rd, a, b readings of 61.5, −1.9, 23.1; 26.9, −3.9, 33.6; and 3.7, −14.9, 0.3 were used to standardize the instrument for pears, carrots, and spinach. This model is obsolete and has been replaced by the automatic unit.

4) Hunterlab D 25 Colorimeter (Hunterlab Associates, Briar Ridge Road, McLean, Virginia). The instrument was set up with a circular 2-in. opening and standardized against a tile with respective L, a, and b readings of 19.2, −12.2, 0.2, and 71.4, 6.9, 29.1 for spinach and pears.

5) Photovolt Photoelectric Reflection Meter, Model 610 (Photovolt Corp., New York 16, N. Y.). The instrument was operated by the suppressed-zero method, using Munsell cards 10 YR 8/5 and 5 R 3/4 for the light and dark standards.

6) Spectronic 20 Color Analyzer (Bausch and Lomb Optical Corp., Rochester, N. Y.). This instrument was standardized against the built-in standard in the cover of the exposure unit. For calculation purposes, the cover was standardized against a Vitrolite glass calibrated by the Nat. Bureau of Standards against magnesium oxide for a Beckman DU spectrophotometer. A rubber guard was placed over the cuvette on the exposure port in such a manner as to cover the top and sides of the cuvette completely in order to eliminate extraneous light.

All instruments were operated according to the manufacturers' instructions in what was considered to be routine fashion without regard to special precautions or manipulations which might be expected to make the instruments more sensitive or more reproducible.

Data from all instruments were converted to C.I.E. x, y, Y values referred to MgO as a standard.

RESULTS AND DISCUSSION

Determination of "just noticeable color differences." A number of samples of carrots were selected by one operator to be fairly similar in color. Each group, comprising 10 samples, was submitted to the panel, which was asked to rank the samples in order from yellowest to most orange. The rankings were analyzed by the method of Krum (1955), and one group showed a significant break in the rankings. Four samples at one end were not significantly different, and were designated as the "dark" group. The other 6 samples, which were similar but different from the first 4, were called the "light" group. Each jar was

Table 1. Color values for two visibly different carrot samples.

Instrument	Chromaticity coordinates					
	Dark group			Light group		
	x	y	Y	x	y	Y
Color-Eye colorimeter	0.5323[a]	0.4221	13.53	0.5288	0.4270	14.40
Colormaster Differential Colorimeter	0.5448	0.4164	14.27	0.5381	0.4220	15.50
Gardner C.&C.D. meter	0.5381	0.4104	14.52	0.5313	0.4143	15.31
Photovolt reflection meter	0.4327	0.3899	14.13	0.4681	0.4170	14.95
Spectronic 20 Color Analyzer	0.4463	0.3973	15.49	0.4507	0.3993	16.17

[a] Each datum represents the average of individual readings on 4 jars for the dark group, and 6 jars for the light group.

opened and measured on 5 colorimeters. The color values (Table 1) from each instrument differed considerably. This was to be expected in view of the possible errors in calibration, non-uniform color spacings for each color solid, differences in viewing geometry, etc. This in itself is not a serious disadvantage in reflection colorimetry, but it serves to emphasize the conclusion of Little *et al.* (1958) that if data from several types of instruments are to be compared, some system of calibration between instruments will have to be used.

The color differences between the light and dark carrot samples were calculated in terms of 5 color-difference equations (Table 2). All 5 gave different values, which were generally but not always in the same order. It is apparent that one can obtain a larger or smaller color difference merely by choosing an appropriate instrument and a method of calculation. The various formulas were developed for materials other than food, and the weightings will probably have to be adjusted for use with food products. If this proves to be the case, the proper application of color-difference formulas to foodstuffs will require considerable research to develop the appropriate weightings for each foodstuff.

The color differences calculated for the 2 groups of carrots were fairly close to the visual threshold, but this was a rather crude method of obtaining a JND. A more accurate method would be to use samples where the color differences could be more accurately controlled. This was done by selecting samples of different colors and blending the two in known proportions. In other experiments, samples were blended

Table 2. Color differences between the light and dark carrot samples calculated by five methods.

Color-difference method	Color-difference units				
	Color-Eye colorimeter	Colormaster Differential Colorimeter	Gardner C.&C.D. meter	Photovolt reflection meter	Spectronic 20 Color Analyzer
Hunter "alpha-beta" chromaticity method[a]	1.74	1.67	2.01	2.78	1.32
Scofield-Hunter method[b]	1.41	2.85	2.16	5.51	2.97
Adams chromatic value[c]	2.85	2.92	2.37	6.31	2.25
Davidson-Hanlon charts[d]	2.8	6.5	5.7	19.4	2.2
Simon-Goodwin charts[e]	4.6	6.3	5.5	15.5	4.0

[a] Equation 31 in Judd (1952).
[b] Equation 32, ibid.
[c] Equation 34, ibid.
[d] Davidson and Hanlon (1955).
[e] Simon and Goodwin (1957).

Table 3. Effect of dilution on the color of pureed spinach as measured with a Colormaster Differential Colorimeter.

Sample % water added	Instrument readings			Chromaticity coordinates			Delta-E [a]
	G	R	B	Y	x	y	
0	8.60	10.25	2.04	8.60	0.4342	0.4424	.....
10	8.81	10.55	2.17	8.88	0.4320	0.4409	0.70
20	9.20	10.92	2.22	9.00	0.4323	0.4419	1.33*
25	9.22	10.92	2.18	9.22	0.4323	0.4419	1.57*
30	9.33	11.07	2.22	9.33	0.4326	0.4430	1.65*
40	9.51	11.28	2.35	9.51	0.4307	0.4405	1.84*
50	9.61	11.34	2.38	9.62	0.4294	0.4413	2.14*

[a] The delta-E value was calculated as the difference between the diluted and undiluted sample, in Adams chromatic value units.

* Visual difference significant at the 0.05 level in a triangle test as judged by a panel of 12 judges.

with water. In both series of experiments, the color changes were in all 3 dimensions of Munsell hue, value, and chroma.

A typical experiment with pureed spinach blended with varying proportions of water is illustrated in Table 3. The various blends in baby-food jars were submitted to a panel for visual judgments using a trianglar difference test. In this way, the delta-E values that represented significant visual differences could be determined. Table 4 shows JND data, obtained by blending and dilution for pureed spinach, carrots, and pears expressed in delta-E units calculated by the Adams chromatic-value equation. The JND data indicate the over-all color difference but do not give any indication of the nature of the color change. In order to do this, data from experiments 1, 3, 6, 10, 12, and 14 were plotted on I.C.I. x, y charts superimposed on Munsell hue and chroma lines (Fig. 1 and 2). The spinach samples had a re-

Table 4. Magnitude of "just noticeable color differences" for pureed spinach, carrots, and pears, in Delta-E (Adams chromatic-value units).

Experiment no.	Just noticeable color difference		Range of color differences presented to panel	
	Color-Eye colorimeter	Colormaster Differential colorimeter	Color-Eye colorimeter	Colormaster Differential Colorimeter
Spinach				
1. Blending	<1.15	<0.73	1.15 to 2.72	0.73 to 1.69
2. Blending	<2.22	<2.45	2.20 to 4.20	2.45 to 3.69
3. Dilution	2.21 to 2.77	0.70 to 1.33	2.21 to 3.61	0.70 to 2.14
4. Dilution	approx. 1.19	approx. 0.63	0.61 to 1.41	0.59 to 1.55
5. Dilution	0.83 to 1.33	0.40 to 1.22	0.83 to 2.32	0.40 to 1.80
Carrots				
6. Blending	0.79 to 1.15	0.45 to 1.15	0.79 to 1.65	0.45 to 1.81
7. Blending	<2.69	<0.69	2.69 to 4.06	0.69 to 2.31
8. Blending	<1.69	<0.64	0.68 to 2.57	0.64 to 1.81
9. Dilution	1.81 to 2.54	1.33 to 1.67	1.04 to 4.75	0.59 to 3.33
10. Dilution	2.19 to 2.97	approx. 1.21	1.50 to 8.82	0.80 to 4.07
Pears				
11. Blending	>1.23	>1.30	0.79 to 1.23	1.11 to 1.30
12. Blending	approx. 0.5	approx. 0.5	0.53 to 2.02	0.11 to 1.40
13. Blending	>2.08	>1.25	1.49 to 2.08	0.93 to 1.25
14. Dilution	1.10 to 2.44	1.12 to 1.99	1.10 to 5.73	0.60 to 3.38
15. Dilution	1.44 to 1.71	0.81 to 0.83	1.44 to 5.33	0.81 to 3.58

flectance range of 5.8-10.2 as measured with the Colormaster; therefore, a Munsell chart at value 3 corresponding to 6.55% reflectance was chosen. The carrot and pear samples had respective reflectance ranges of 12.2-15.9 and 21.4-23.8; therefore, Munsell value 4 was chosen for the carrots and 5 for the pears. Data from the other 9 experiments, being similar, were not reproduced here.

The blending and dilution experiments were set up in the hope that the color changes would be much larger in one of the three color attributes. This was partially true since in the spinach blending experiments the range of color differences indicated in Table 4 clustered about an area within

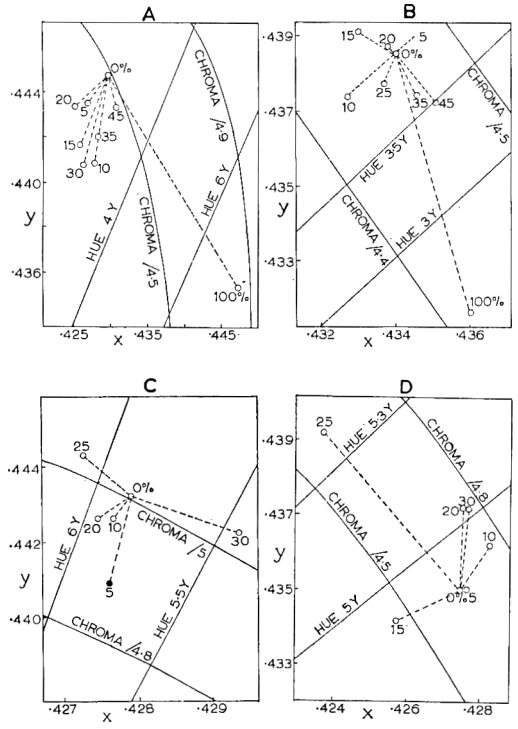

Fig. 1. Color data for spinach samples expressed in I.C.I. x, y coordinates superimposed on Munsell constant-hue and -chroma lines. The solid dots indicate samples not visually different from the 0% sample at the 5% level of significance. The hollow dots represent visually different samples. A) Intermediate colors obtained by blending 2 samples as measured with a Color-Eye (Expt. No. 1, Table 4); B) intermediate colors obtained by blending as measured with a Colorimeter (Expt. No. 1); C) intermediate colors obtained by diluting a sample of spinach with water as measured with a Color-Eye (Expt. No. 3); D) intermediate colors obtained by dilution as measured with a Colormaster (Expt. No. 3).

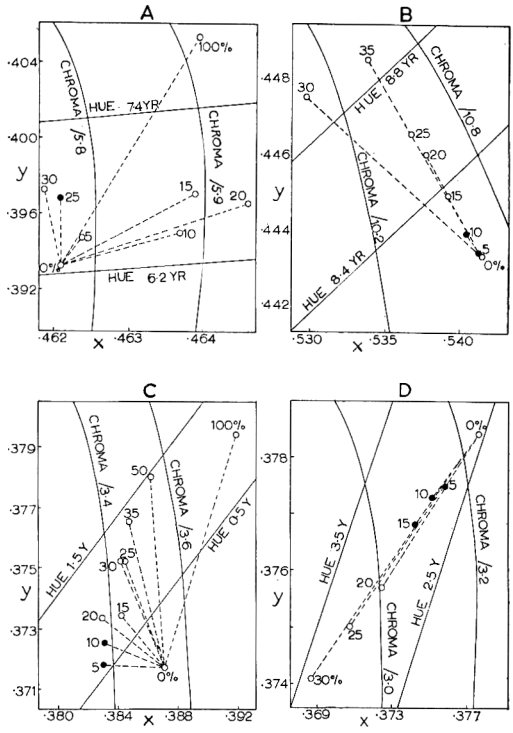

Fig. 2. Color data obtained with a Colormaster for carrot and pear samples expressed in I.C.I. x, y coordinates superimposed on Munsell constant-hue and -chroma lines. The solid dots indicate samples not visually different from the 0% sample at the 5% level of significance. The hollow dots represent visually different samples. A) Intermediate colors obtained by blending 2 samples of carrots. (Expt. No. 6); B) intermediate colors obtained by diluting a sample of carrots with water (Expt. No. 10); C) intermediate colors obtained by blending 2 samples of pears (Expt. No. 12); D) intermediate colors obtained by diluting a sample of pears with water (Expt. No. 14).

a radius of 0.5 delta hue and 0.05 delta chroma (Fig. 1). Delta hue and delta chroma were used to designate the distance from the standard sample to the blends in a direction perpendicular to the constant-hue and constant-chroma lines. For the dilution experiments with spinach, the delta-hue and delta-chroma changes were respectively as much as 2 and 0.2 units. Small changes in chroma were to be expected with such dark samples. The JND values for the blended spinach samples were below 1.15 delta-E units as measured with the Color-Eye, and 0.73 units for the Colormaster. The dilution studies showed that the JND was be-

tween 0.83 and 2.77 for the Color-Eye and 0.40 to 1.22 units for the Colormaster.

With the range of color differences indicated for carrots in Table 4, the blended samples showed a range of 0.6 delta-hue units and 0.2 delta-chroma units. The diluted samples indicated respective delta-hue and -chroma figures of approximately 0.8 and 0.4. The JND values for the blended carrots were 0.79–1.15 for the Color-Eye and 0.45-1.15 for the Colormaster. For the diluted samples the values were 1.81–2.97 and 1.33–1.67 for the two instruments.

The experiments with pears pointed out the well known differences in sensitivity of the human eye to changes in hue or lightness. In Expt. 11 of Table 4, a light sample was mixed with a dark standard, and in the resultant blends the changes were mainly in the lightness attribute, since the delta-hue and delta-chroma figures were of the order of 0.1 unit. In this case the JND was larger than 1.23 for the Color-Eye and 1.30 for the Colormaster. In Expt. 12, the sample used to mix with the standard was slightly brownish and the resultant blends had delta-hue differences of 1.5 units. With these blends the JND was approximately 0.5 unit for both instruments. For the diluted samples the JND values were 1.10–2.44 for the Color-Eye and 0.81–1.99 for the Colormaster.

The above experiments to determine the JND values for spinach, carrots, and pears were carried out under conditions for fairly good discrimination of color differences. Such factors as large field illumination, good level of illumination, binocular vision, and smooth surfaces were all present. The JND values determined under these conditions were approximately 1–2.5 delta-E units. Consequently, it is probably safe to assume that color differences of this order for pureed spinach, carrots, and pears will be imperceptible under ordinary conditions of merchandising.

Determination of instrument repeatability. If we assume that the JND is approximately 1–2 Adams chromatic-value units for pureed spinach, carrots, and pears, it is of importance to determine how the repeatability of the colorimeters compares to this figure.

The repeatability studies were conducted on 30 samples of carrots and 10 each for spinach and pears. All samples were measured in duplicate on each instrument by each of 2 operators in the same time sequence in order to minimize drift and sample change problems. Each instrument was turned on at least 2 hr before use and restandardized every 3 hr. The delta-E values were calculated between duplicate readings on each sample.

Table 5 shows the delta-E values for repeatability for 6 instruments using carrots, spinach, and pears. The delta-E values for carrots were of the same order of magnitude as the JND values, whereas the respective values for spinach and pears were approximately ½ and ¼ that of the JND.

A method of determining repeatability on samples of baby food that have obviously been removed from the container is open

Table 5. Instrument repeatability in color measurement of pureed food.

Product	Delta-E (Adams chromatic-value units)					
	Color-Eye colorimeter	Colormaster Differential Colorimeter	Gardner C.&C.D. meter	Hunter D_{25} colorimeter	Photovolt reflection meter	Spectronic 20 Color Analyzer
Carrots						
Lot No. 1	1.45[a]	2.67	1.15	.....	2.09	1.17
Lot No. 2	1.61	1.51	2.05	.....	1.61	1.27
Lot No. 3	2.08	2.02	2.06	.....	1.93	1.28
Spinach	0.75	0.33	0.82	0.68	.....	.....
Pears	0.31	0.29	0.34	0.43	.....	.....

[a] Each datum represents an average obtained from 20 values of delta-E, calculated as the difference between duplicate readings for each of 2 operators.

Table 6. Changes in color of pureed food during measurement.

Time (minutes)	Delta-E (Adams chromatic-value units)		
	Carrots	Spinach	Pears
30	0.03[a]	0.74	0.40
60	0.18	0.40	0.40
90	0.53	0.52	0.31
120	0.65	0.47	0.31
150	0.65	0.59	0.23
180	0.67	0.75	0.34
Av.	0.50	0.58	0.33

[a] Each datum represents an average of 4 delta-E values calculated from data obtained with a Colormaster Differential Colorimeter calibrated before each reading. The delta-E value was calculated as the difference between the initial reading and that at the indicated time.

to the objection that the food sample may change during measurement. Little *et al.* (1958) reported that mixtures of carrot and pea puree showed a change in color after the container was opened. This was investigated for the carrot, spinach, and pear samples used in this work by measuring the color at definite intervals after opening (Table 6). There was little if any indication of a color change during the 3-hr period.

The repeatability values in Table 5 have several sources of variance. One is the normal variability associated with the mechanics of measurement, another is the possible color change of the product, and a third could be instrument drift during measurement. The second one was apparently very small in this work. The third source was tested with Munsell cards in the expectation that the product color change with time would be eliminated (Table 7). The values for the Color-Eye and the Colormaster were of the same order of magnitude as those reported in Table 6, and for spinach and pears in Table 5. The other instruments showed greater drift, but this could be reduced by more frequent standardization. For the Color-Eye and the Colormaster, the over-all repeatability cannot be reduced by adjustments for sample change or instrument drift, at least with spinach and pears. The reason for the higher repeatability figures for carrots is not apparent at present.

The use of a colorimeter to obtain JND values for pureed spinach and pears is probably justified because the repeatability of at least 2 instruments was lower than the JND values. For carrots, the 2 are of the same order of magnitude; therefore there is little to be gained in attempting to determine JND values in this manner more accurately than those in Table 5. The plots in Fig. 1 and 2 suggest a scatter diagram in several instances, which indicates that the order of differences under investigation is close to the repeatability of the instruments. It is apparent that a more accurate method of measuring color differences would be desirable if one wishes to determine JND values more accurately.

Table 7. Instrument drift during measurement.

Minutes after calibration	Delta-E [a] (Adams chromatic-value units)				
	Color-Eye colorimeter	Colormaster Differential Colorimeter	Gardner C.&C.D. meter	Photovolt reflection meter	Spectronic 20 Color Analyzer
Run No. 1					
30	0.60	0.36	1.18	2.67	0.10
60	0.60	0.30	1.15	4.33	0.85
90	0.71	0.30	0.71	7.05	4.53
120	0.59	0.52	3.73	3.37	1.56
150	0.68	0.36	2.04	3.38	3.65
180	0.74	0.52	3.78	3.45	2.35
Av.	0.65	0.39	2.28	4.04	2.17
Run No. 2					
180	0.63	0.50	6.01	2.90	2.50

[a] Delta-E was calculated as the difference between the initial reading and that at the stated time interval on a Munsell card (10 YR 5/10, 6473). All instruments had been turned on for at least 2 hr before measurement.

It may be concluded that several of the colorimeters tested in this work could be used to detect color differences in pureed spinach, carrots, and pears at least as well as an average visual panel. This does not mean to imply that a highly trained panel operating under ideal conditions could not do a much better job of judging color differences.

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RESEARCH NOTE

The Effect of Oxidizing and Reducing Aging Media on the Tenderness of Excised Chicken Muscle[a]

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Data accumulated in the literature indicate that tenderness of meat depends on changes that occur during the development of rigor, perhaps more than on any other factor. De Fremery and Pool (1960), investigating the biochemical changes in chicken muscle related to rigor, showed that treatments that increase the rate of ATP breakdown in the muscle produce less tender meat. The interpretation was that quick ATP loss produces a more rapid onset of rigor. The more rapid the onset of rigor, the tougher the meat. The reason for this phenomenon was not explained. The biochemical changes associated with the resolution of rigor and their contribution to tenderness of meat upon continuous aging are relatively unknown. There is no general agreement among workers on the nature of the changes that occur during the resolution of rigor and prolonged aging. None of the chemical reactions that, *prima facie,* could be associated with tenderness upon continuous aging, such as proteolysis, changes in the connective tissue, dissolution of actomyosin, and increased hydration of proteins, have been proved conclusively.

In an attempt to further the understanding of the factors that contribute to changes in tenderness of meat during aging, the effects of certain oxidizing and reducing aging media on tenderness of excised chicken muscles were investigated. The results and the suggested mechanisms involved are reported here.

Part I. The pectoralis major muscles of six adult chickens were used. After slaughter and evisceration, the pectoralis major muscles were removed, and each muscle of a pair (left or right) was put into a different flask containing the appropriate aging medium: water, 0.2*M* potassium iodate, or 0.2*M* sodium hydrosulfite. The flasks were stored 18 hr. at 1°C and at room temperature for an additional 2 hr. The muscles were wrapped with aluminum foil and cooked 80 min in boiling water. Rectangular sections (1 cm^2 cross-section) were then cut parallel to the muscle fibers, and shear force measurements taken with a modified Warner-Bratzler shear apparatus (Spencer *et. al.,* 1962).

Highly significant differences ($P < 0.005$) were observed between muscles which were aged in iodate or water (Table 1) and between muscles aged in iodate or hydrosulfite (Table 2). The iodate caused the muscles to be considerably tougher. Nonsignificant differences were observed between hydrosulfite and water treatments under the conditions of this experiment (Table 3).

The results obtained indicated that certain oxidation reactions may play an important role in chicken meat tenderization during post-mortem aging. Redox agents acting in the aging media are important factors in post-mortem tenderization. Dodge and Stadelman (1959), studying the effect of the aging media on tenderness, found differences in tenderness scores between carcasses aged in water and carcasses aged in air to be highly significant. The adverse changes in the tenderness of the muscles found in that study can be attributed to air oxidation rather than dehydration.

The oxidation effect of iodate and other oxidizing agents on the labile sulfhydryl groups (–SH) of cysteine, glutathione, and thioglycolate to form nonlabile disulfide bonds (–S–S–) with a small amount of sulfinates ($-S_2H$) and sulfonates ($-SO_3H$) has been demonstrated by Hird and Yates (1961). The results of the experiments presented here suggest a similar oxidative reaction by iodate on the protein of chicken muscles. These reactions and similar oxidation reactions that occur during the early stages of aging may affect:

[a] Scientific paper No. 2163, Washington Agricultural Experiment Station, Pullman. Project 1515.

Table 1. Effects of potassium iodate aging medium versus water aging medium on tenderness of pectoralis major of chickens.

Trial	Treatment	No. of cuts	Shear force		Pooled t
			Variance	Mean (lb/cm ^{2})	
1	Potassium iodate	10	2.78	6.83	5.10[a]
	Water	10	2.19	3.04	
2	Potassium iodate	12	7.04	6.13	3.87[a]
	Water	12	0.58	2.88	

[a] $P < 0.005$.

Table 2. Effect of potassium iodate aging medium versus sodium hydrosulfite aging medium on tenderness of pectoralis major muscles of chickens.

Trial	Treatment	No. of cuts	Shear force		Pooled t
			Variance	Mean (lb/cm ^{2})	
1	Potassium iodate	12	3.33	9.31	7.69[a]
	Sodium hydrosulfite	12	1.08	4.44	
2	Potassium iodate	14	6.81	7.54	4.14[a]
	Sodium hydrosulfite	14	0.47	4.43	

[a] $P < 0.005$.

Table 3. Effect of sodium hydrosulfite aging medium versus water aging medium on tenderness of pectoralis major muscles of chickens.

Trial	Treatment	No. of cuts	Shear force		Pooled t
			Variance	Mean (lb/cm ^{2})	
1	Sodium hydrosulfite	12	7.65	7.46	1.39[a]
	Water	12	2.11	8.77	
2	Sodium hydrosulfite	12	1.99	3.82	0.45[a]
	Water	12	1.61	3.56	

[a] $P > 0.05$.

Table 4. Effect of sodium sulfite versus water treatment on tenderness of pectoralis major muscles of chickens.

Trial	Treatment	No. of cuts	Shear force		Pooled t
			Variance	Mean (lb/cm²)	
1	Sodium sulfite	10	0.17	1.19	
					12.7[a]
	Water	10	0.29	4.05	
2	Sodium Sulfite	10	0.08	0.68	
					6.4[a]
	Water	10	0.72	2.60	
3	Sodium sulfite	10	0.14	1.55	
					45.0[a]
	Water	10	1.06	7.04	

[a] $P < 0.005$.

1) The sulfhydryl groups of adjacent protein molecules, oxidizing them to produce intermolecular disulfide bonds;

2) The sulfhydryl groups of the same molecule, producing intramolecular disulfide bonds;

3) The sulfhydryl groups, oxidizing them to the sulfinic and sulfonic acids, thus preventing possible formation of disulfide bonds (this to a much lesser extent).

Part II. In this experiment excision of the muscles was delayed for 2 hr after slaughter, at which time the birds were obviously in rigor. One muscle of a pair was put into a flask containing 0.2*M* sodium sulfite and the other in water, and aged 6 hr at room temperature. The muscles were wrapped with aluminum foil, cooked 65 min in boiling water, and tenderness measurements taken.

Highly significant differences ($P < 0.005$) were observed between the forces required to shear muscles treated with sodium sulfite and those treated with water. The sodium sulfite-treated muscles were more tender (Table 4). The ability of the sulfites to cleave disulfide bonds has been demonstrated by several investigators (Cecil and McPhee, 1959). The probable action of sodium sulfite on meat protein was to reduce the disulfide bonds. The sulfhydryls thus formed were then reoxidized so that the final products were the S-sulfonates.

$$RSSR + SO_3^{=} \rightleftharpoons RS^{-} + RSSO_3^{-}$$

$$\uparrow \quad \downarrow$$

$$\longleftarrow (O) \longrightarrow$$

The results suggest that the changes in tenderness of poultry meat during the resolution of rigor and continuous aging may be due to cleavage or reorientation of intermolecular and/or intramolecular disulfide bonds. Such cleavage of disulfide bonds in the aging meat may be caused by redox enzyme systems originating naturally within the meat. The probable end products of the reaction are sulfonates ($-SO_3H$), with a small amount of sulfinates ($-SO_2H$) due to disulfide-sulfhydryl exchange.

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RESEARCH NOTE

DIRECT GAS CHROMATOGRAPHY OF MILK VAPORS

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This is a report of the successful direct application of gas chromatography to milk vapors. The apparatus, constructed with the help and advice of Drs. J. W. Corse and R. Teranishi, was similar to that described by Buttery and Teranishi (1961) and utilized a McWilliam-Dewar circuit (1958). The chromatograms shown in this report were run on 1% Apiezon N on 80–100-mesh Chromosorb W at 35°C. The carrier gas, high-purity water-pumped nitrogen, was bubbled through a water-filled scrubbing tube before being conducted to the instrument. It was found that results were quite satisfactory, even when dissimilar packings were used in the two columns. In the work reported here, the reference column was packed with 1% LAC 728 on 60–80-mesh chromosorb W. Since injections can be made on either column, relegating the other to the role of a reference column, this offers the advantage of immediate availability of different substrates.

Because the extreme sensitivity of this apparatus readily detects contaminants in laboratory air, it was found necessary to sample in a free air space out of doors. Syringes and glassware were given periodic vacuum-oven treatments. A 125-cc Erlenmeyer flask was completely filled with milk to be tested and about half the milk decanted outside. The flask was stoppered and heated 1 hr at 80°C, and a 1-cc gas sample was withdrawn through the stopper for analysis. As discussed below, the compounds generated by subjecting milk to this heat treatment are evidently not detected by flame ionization. Application of this procedure to redistilled water resulted in peaks whose relative retentions agreed with those of 1 and 2; no other discernible peaks were apparent. Consequently, the relative heights of peaks 1 and 2 are of doubtful significance.

Several determinations were made on each of the samples shown in the accompanying figures. The figures are representative, and reproducibility was good, including results from separate sampling procedures on different aliquots of the same milk.

Fig. 1 reproduces a chromatogram from raw mixed herd milk, and Fig. 2 from the same milk after pasteurization and homogenization. The similarity of the curves demonstrates that the results are highly reproducible, and that they are not influenced by pasteurization and homogenization with conventional HTST equipment. Fig. 4 is a curve for pasteurized homogenized milk produced by the same herd one day later. The differences illustrate day-to-day variations related to the milk supply. The chromatogram of the copper-oxidized sample (Fig. 3) is characterized not only by differences in

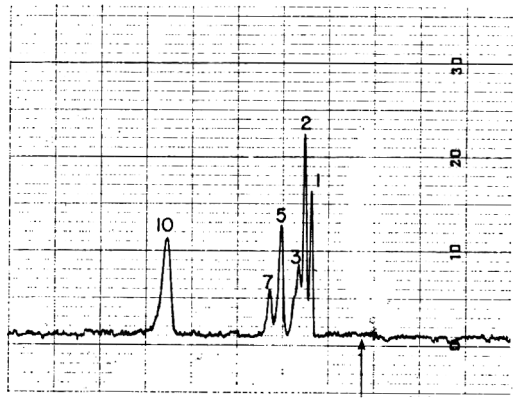

Fig. 1. Chromatogram of 1-cc vapor sample of raw mixed herd milk.

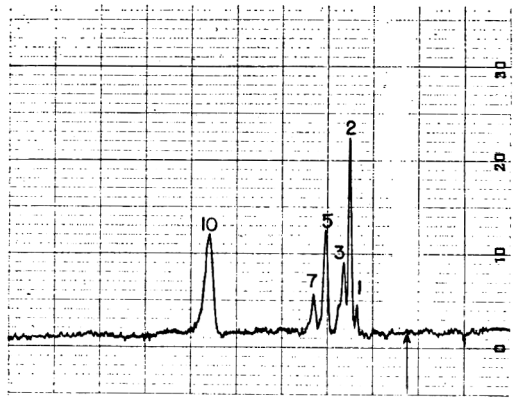

Fig. 2. Chromatogram of 1-cc vapor sample of pasteurized homogenized milk.

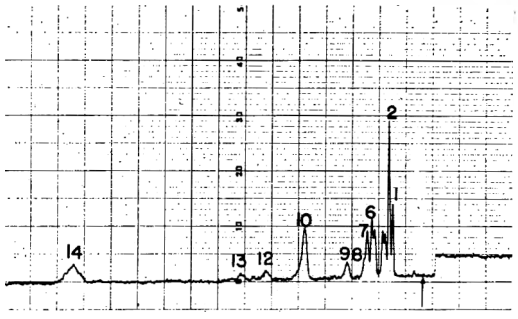

Fig. 3. Chromatogram of 1-cc vapor sample of copper-catalyzed oxidized milk.

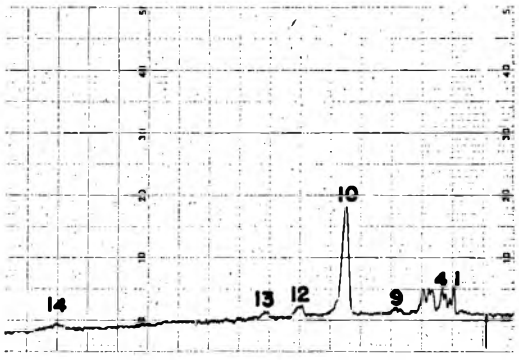

Fig. 6. Chromatogram of 1-cc vapor sample of milk exposed to fluorescent light for 6 hr.

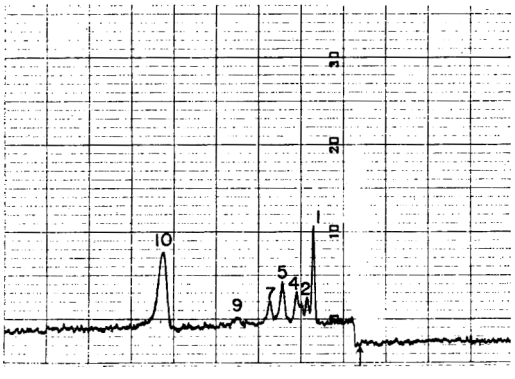

Fig. 4. Chromatogram of 1-cc vapor sample of pasteurized homogenized milk. Control from light exposure experiments.

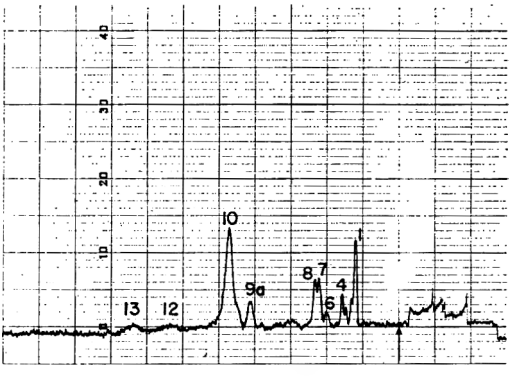

Fig. 7. Chromatogram of 1-cc vapor sample of milk exposed to germicidal lamps.

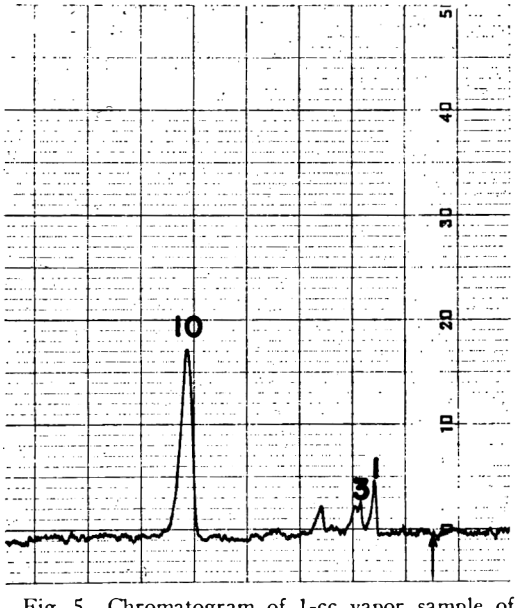

Fig. 5. Chromatogram of 1-cc vapor sample of milk exposed to fluorescent light for 1 hr.

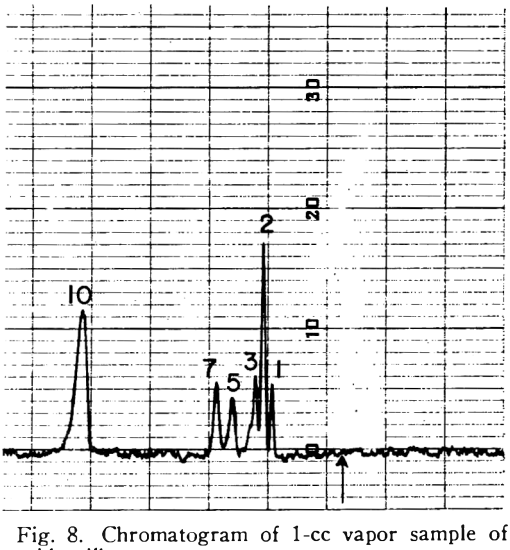

Fig. 8. Chromatogram of 1-cc vapor sample of rancid milk.

the more volatile fraction but by the appearance of at least 3 slower-moving (and probably higher-boiling) components. Figs. 4 to 7 are for the same milk subjected to different light exposures, in order of increasing severity. The volatile components correspond to those in copper-oxidized milk. Relative amounts, however, differed considerably (note the peak sizes, peaks 1 to 8 and 14 being larger, and peak 10 smaller in the copper-oxidized milk). Peaks 9, 12, and 13 were about the same size in both cases.

With the Apiezon column it was not possible to demonstrate differences due to hydrolytic rancidity (Fig. 8), although three separate judges agreed the milk possessed a strong rancid flavor. Under the conditions used to obtain these chromatograms, butyric acid has an exceedingly long retention time. It is probable that a second column, containing an acidic substrate, would be of more use in the detection of rancidity. This is being investigated.

Fig. 9 illustrates the appearance of a new peak, designated 0, a marked increase in peak 7, and to a lesser degree peak 9, in milks possessing an alfalfa feed flavor.

Wynn *et al.* (1960) reported acetone, acetaldehyde, and methyl sulfide to be constituents of the volatile fraction of mixed herd milk. The retentions of peaks 3, 9, and 10 respectively agree with those of acetaldehyde, methyl sulfide, and acetone. Other components were not identified, but will be investigated.

The mechanism of ion formation in the hydrogen flame is not well understood, but apparently demands a carbon-containing molecule. Because no qualitative differences could be detected between cold and hot sampling procedures, even on raw milk, it appears that the major products of this heat treatment are inorganic substances, such as H_2S, which are not detected by flame ionization.

Ultrasensitive flame ionization with use of a water-saturated carrier gas, perhaps modified to include a variety of substrates, permits rapid qualitative and quantitative determination of milk flavors. Because diagnosis of a flavor defect is a prerequisite to searching out and correcting the conditions causing that defect, this approach has a great practical potential. A combination of flavor defects sometimes baffles even trained judges, but an analysis of this type should establish just what defects are contributors in any given case.

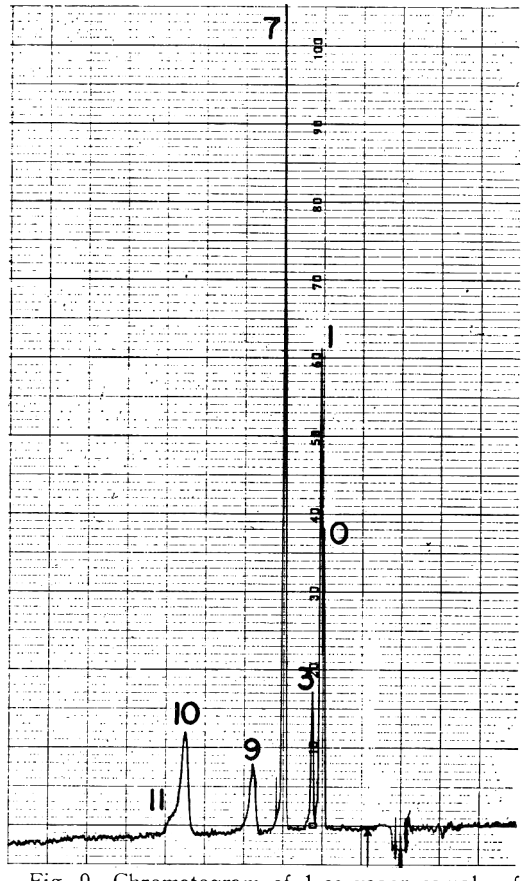

Fig. 9. Chromatogram of 1-cc vapor sample of alfalfa-feed-flavor milk.

These techniques should also allow evaluation of various off-flavor removal devices, such as vacuum pasteurizers. This laboratory is currently engaged in applying these methods to such a study.

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Application of Activation Analysis to the Determination of Trace-Element Concentrations in Meat[a]

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SUMMARY

The concentrations of 27 trace elements were determined in four meats: beef, pork, ham, and chicken. Because the concentrations of most of these elements were expected to be very small, the extremely sensitive method of neutron activation analysis was used in this study. Qualitative analyses were performed for 8 of the elements, and the concentrations of 19 elements were determined quantitatively. The quantitatively measured concentrations varied from ~0.1% for phosphorus to $\sim 10^{-5}$ ppm for cerium. Most data are estimated to be accurate to ±10%. The results demonstrate the applicability of this analytical method to determination of the inorganic constituents in foods and related substances at a constant level of accuracy throughout the concentration ranges of practical interest.

INTRODUCTION

A comprehensive program has been initiated to characterize the elemental constituents in bulk samples of four meats: beef, pork, ham, and chicken. In the first phase of this study, activation analysis has been applied to the determination of 27 trace elements in these meats. The analytical data demonstrate the applicability of this analytical method to determination of the inorganic constituents in foods at a constant level of accuracy throughout the concentration ranges of practical interest.

Many elements normally present as trace constituents in foods are necessary to the maintenance of life and health. For example, calcium and phosphorus are the main components of bone; sodium, potassium, and chlorine help maintain the osmotic pressure of body liquids; and a number of elements, such as iron and manganese, activate hormones. Other elements, such as cobalt, are components of vitamins or other substances essential to a balanced diet.

The recognition of the importance of trace elements in nutrition has led to increasingly stringent requirements for sensitivity and specificity in food analysis. Several analytical methods, such as emission spectroscopy, flame spectrophotometry, colorimetry, amperometric titrations, and activation analysis, are generally useful for trace-element analysis. A comparison of these methods (Meinke, 1955) shows that activation analysis has the best sensitivity for a majority of the elements. Activation analysis has been used for the determination of specific elements in animal tissue and vegetation (Bowen, 1956, 1959a,b; Gibbons, 1958; Goette, 1955; Harrison, 1955; Kruger, 1962; Smales, 1952; Smith, 1959). The demonstrated versatility of activation analysis and its intrinsic sensitivity for most elements led to its choice for this application.

Activation analysis has been defined (Koch, 1960) as a method of determining the concentration of an element in a matrix by measuring the characteristic radiations emitted by a radioactive nuclide resulting from a specific nuclear reaction in the trace element. This radioactive reaction product, or activation product, possesses a unique combination of physical, chemical, and nuclear properties that provides the necessary specificity for its identification and measurement.

The general method of neutron activation analysis involves irradiation of the samples

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to be analyzed in a neutron flux, such as is found in a nuclear reactor, to produce the desired activation products in sufficient quantity for accurate measurement. After irradiation, the samples are analyzed for the radioisotopes formed in each element of interest. This analysis involves chemical separation and purification of the activation product and determination of its disintegration rate by measurement of its characteristic beta or gamma radiations.

During the irradiation, neutrons are absorbed by nuclei of some of the atoms in the sample, producing different nuclei. The neutron absorption is accompanied by emission of a second nuclear particle or a quantum of electromagnetic radiation. The most common reaction involving reactor neutrons is the (n, γ) reaction in which the neutron is absorbed in the nucleus, followed by emission of a high-energy gamma ray. This reaction produces an isotope of the same element as the reactant atom. The product isotope is often radioactive. Hence, it is termed an "activation product."

The production (Koch, 1960) of an activation product proceeds ideally according to equation 1:

$$D(t) = \phi n \sigma (1 - e^{-\lambda t}) \qquad [1]$$

where $D(t)$ = the rate of decay of the radioactive species in the sample after irradiation time, t, disintegrations per second;
ϕ = the neutron flux, neutrons/cm^2-sec
n = the number of atoms of the target isotope in the irradiation sample;
σ = the activation cross-section, cm^2/atom;
λ = the decay constant of the nuclide, sec^{-1}; and
t = the irradiation time, sec.

Low-energy or thermal neutrons induce the (n, γ) reaction primarily. However, higher-energy, or fast, neutrons are also present in reactors. The net effect of the interactions of these neutrons may be the transmutation of an atom of one element to an atom of a different element, since charged particles such as protons or alpha particles may be produced in these reactions. Thus, if an (n, p) reaction, in which a proton is produced, occurs in an atom having atomic number Z, an isotope of the element Z-1 is produced.

Activation analyses are usually performed by a comparative method. In this method, comparator samples containing known quantities of the trace elements to be determined, are irradiated and analyzed concurrently with the matrix sample. The concentration of a trace element in the matrix is then calculated using equation 2:

$$m_s = \frac{m_c D(t)_s}{D(t)_c} \qquad [2]$$

where m_s and m_c are the respective masses of the trace element in the matrix sample and comparator, and $D(t)_s$ and $D(t)_c$ are the respective disintegration rates of the activation product.

The performance of a neutron activation analysis requires a set of six operations (Koch, 1960): 1) the selection of an appropriate neutron-induced activation reaction; 2) the specification of comparator samples; 3) the choice of a suitable irradiation facility; 4) the preparation of the samples for irradiation; and 5) the irradiation and 6) the post-irradiation analyses. Consideration was given to the requirements imposed by each of these operations in delineating the scheme of analysis used in this work. A description of these considerations and of their application in this program is presented.

Selection of activation reactions. The first operation in performing activation analysis is the selection of the optimum activation reaction. This selection involves an evaluation of: 1) the suitability of the activation product, including the feasibility of performing the post-irradiation assays; and 2) the extent to which interfering reactions in the matrix may produce or consume the activation product to be assayed. Since alternative neutron activation reactions provide analytical sensitivity sufficient for most applications, sensitivity is seldom an important factor in this selection.

The basic requirement for a useful activation product is that it be radioactive, with

a half-life long enough to permit the post-irradiation measurements. The minimum useful half-life is dependent primarily on the complexity of the chemical separations. In special cases where direct measurement of the activation product can be made in the sample, a half-life of the order of minutes may be acceptable.

Activation products that are gases or may be present in volatile forms should be avoided. If such species must be used, precautions are required to prevent their release from the sample during irradiation or as a result of chemical processing.

An interfering reaction has been defined (Koch, 1960) as a nuclear reaction in a constituent of the matrix, other than a stable isotope of the trace element to be determined, that produces or consumes the activation product to be measured. An interfering reaction may significantly affect the measured quantity of the activation product and produce an erroneous result. There are several possible sources of interference, but only two general types warranted serious consideration in this work.

The first type of interference involves nuclear reactions resulting in the production of charged particles. For example, if the trace element having atomic number Z is to be determined, interference may result from an (n,p) reaction with element $Z + 1$ or from an (n, α) reaction with element $Z + 2$, either of which produces the desired activation product. The effective cross-sections for the (n, p) and (n, α) reactions are generally several orders of magnitude less than those for the (n, γ) reactions. Therefore, this interference is usually very small if the two elements have similar concentrations. It may be important, however, if the trace element is present only in exceptionally small concentrations.

The second type of interference involves the production of the desired activation product by neutron-induced fission reactions, primarily in uranium. During the fission process, the uranium nucleus is split into two fragments, producing radioactive isotopes of the entire spectrum of elements from zinc to terbium. Since many of the useful activation products of these elements are also fission products, this type of interference is potentially widespread. Uranium may interfere with the determination of many of these elements, even when it is present in only trace concentrations.

Since the concentrations of many of the elements in the meats to be analyzed in this investigation were expected to be very small, special attention was directed to avoiding or compensating for potential interfering reactions. For some elements, alternative isotopes are available for which interference does not occur. For example, the 65-day Sr^{85} and 11.6-day Ba^{131} isotopes were chosen for use in analysis for strontium and barium, rather than the 54-day Sr^{89} and 85-min Ba^{139}, because the latter two isotopes are formed in uranium fission.

For some of the elements of interest, interfering reactions could not be avoided. Therefore, data were required to determine the extent of the interference and to correct for it. The necessary data are obtained by analysis for the sought activation product in a comparator sample of the interfering element, and by determination of the interfering element in the matrix. The quantity of the activation product in the matrix that is due to the interfering reaction is then calculated using equation 2.

An important example of this type of interference occurs in analysis for phosphorus and sulfur. The pertinent section of the Chart of the Nuclides (Stehn and Clancy, 1956), presented in Fig. 1, shows the several possible neutron-induced reactions in these elements and the modes of interference with sulfur and phosphorus analyses. From Fig. 1 it is seen that (n, γ) reactions in chlorine yield the 3×10^5-year Cl^{36} and the 37-min Cl^{38}, and the (n, p) reaction in Cl^{35} yields

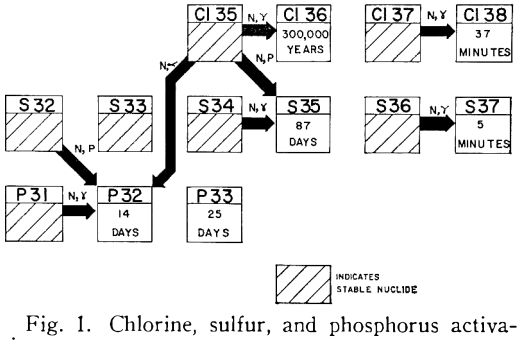

Fig. 1. Chlorine, sulfur, and phosphorus activation.

Table 1. Data for activation products and interfering reactions.

Element	Activation product		Radiation measured	Interfering reaction
Phosphorus	14.2-d	P^{32}	1.707 Mev β^-	$S^{32}(n, p)$, $Cl^{35}(n, \alpha)$
Sulfur	87-d	S^{35}	0.1617 Mev β^-	$Cl^{35}(n, p)$
Chlorine	3.0×10^5-y	Cl^{36}	0.714 Mev β^-	None
Scandium	83.9-d	Sc^{46}	0.885 Mev γ	$Ti^{46}(n, p)$
Iron	45.1-d	Fe^{59}	1.098 Mev γ	$Co^{58}(n, p)$
Cobalt	5.24-y	Co^{60}	1.1728 Mev γ	$Ni^{60}(n, p)$
Zinc	245-d	Zn^{65}	1.119 Mev γ	None
Selenium	127-d	Se^{75}	0.402 Mev γ	None
Rubidium	18.66-d	Rb^{86}	1.079 Mev γ	$Sr^{86}(n, p)$
Strontium	64-d	Sr^{85}	0.513 Mev γ	None
Zirconium	65-d	Zr^{95}	0.723, 0.756 Mev γ	U, Th fission
Ruthenium	39.8-d	Ru^{103}	Multiple β^-	U, Th fission
Palladium	17-d	Pd^{109}	Multiple γ	$Cd^{106}(n, \alpha)$
Indium	49-d	In^{114m}	0.190 Mev γ	None
Tin	119-d	Sn^{113}	0.393 Mev γ	$Sb^{123}(n, p)$
Tellurium	104-d	Te^{123m}	0.158 Mev γ	None
Cesium	2.07-y	Cs^{134}	0.796 Mev γ	$Ba^{134}(n, p)$
Barium	11.5-d	Ba^{131}	0.214 Mev γ	None
Cerium	32.5-d	Ce^{141}	Multiple γ	$Pr^{141}(n, p)$ U, Th fission
Hafnium	70-d	Hf^{175}	0.089 Mev γ	None
Iridium	74.4-d	Ir^{192}	Multiple β^-	$Pt^{192}(n, p)$
Platinum	4.3-d	Pt^{193m}	0.136 Mev γ	None
Uranium-238	2.346-d	Np^{239}	Multiple β^-	None
Uranium-235	12.8-d	Ba^{140}	Multiple β^- (La^{140})	None

Table 2. Data for comparator samples.

Trace element	Primary comparator	Secondary comparator	Typical wt. of element in prim. comp. (mg)
Phosphorus	$Mg_2P_2O_7$	S, NaCl	2.8
Sulfur	S	NaCl	23
Chlorine	NaCl	.....	10.4
Scandium	$Sc(NO_3)_3$	Ti	0.001
Iron	Fe	Al—0.1% Co alloy	9.8
Cobalt	Al—0.1% Co alloy	Ni	0.01
Zinc	Zn	.....	11
Selenium	Se	.....	1.0
Rubidium	RbCl	$Sr(NO_3)_2$	1.0
Strontium	$Sr(NO_3)_2$	.....	20
Zirconium	$ZrO(NO_3)_2 \cdot 2H_2O$	.....	10.3
Indium	In	Sn	0.12
Tin	Sn	Sb	22
Tellurium	Te	.....	10
Cesium	$CsNO_3$	$Ba(NO_3)_2$	0.08
Barium	$Ba(NO_3)_2$	.....	11
Hafnium	Hf	.....	0.12
Uranium-235	Enriched U	.....	0.01
Uranium-238	Natural U_3O_8	.....	1.0

87-day S^{35}. The latter nuclide is also formed by the (n, γ) reaction in S^{34}. Since the only other (n, γ) activation reaction in sulfur yields the very short-lived S^{37}, measurement of S^{35} is required for neutron activation analysis for sulfur in meat ash.

Similarly, the only (n, γ) activation product in phosphorus is 14.2-day P^{32}, which is also produced by the (n, p) reaction in S^{32}. Therefore, the mutual interference of these elements is unavoidable, and an experimental study was required to determine the extent of the interference. This problem is discussed in detail in Appendix I.

A review of the above criteria for the nineteen elements to be analyzed quantitatively led to selection of the activation products shown in column 2 of Table 1. Column 3 of the table presents the radiation that was measured, and column 4 shows the potential interference for the respective elements.

Selection of comparators. Comparator samples are required for each trace element to be determined. If interfering reactions may occur, secondary comparators, containing the interfering element, are also utilized. In each case, the comparators are prepared from known amounts of the respective elements or of their compounds. If a compound is used, it must exhibit stoichiometric and radiolytic stability. In addition, care must be taken to avoid local neutron flux disturbances by comparators for elements having large cross-sections for neutron absorption.

Table 2 summarizes the chemical form and typical weights of the comparator samples used in this program. The weights were chosen to limit the attenuation of the neutron flux by the sample to 1%.

EXPERIMENTAL PROGRAM

Sampling and sample preparation. Since the bulk meat samples were to be representative of portions consumed by humans, all bones, cartilage, glands, and other inedible matter were removed. To obtain statistically significant samples, each meat was ground through a ½–1-in. plate, followed by a second grinding through a ⅛–¼-in. plate. The meat was then mixed by machine into a homogeneous mass, packaged, and kept frozen until used.

Weighed portions of the homogenized meats were ashed to concentrate the inorganic constituents into samples convenient for irradiation. The samples were partially ashed by slow combustion in large stainless-steel vessels and then ignited in large porcelain crucibles at 800°C. In removing the partially ashed residues from the steel vessels, care was taken to avoid their contamination with traces of metal. The ratios of ash to bulk weights varied from approximately 0.5% to 2%, as shown in Table 3.

Table 3. Weights of bulk and ashed meats.

Food	Bulk wt (kg)	Ashed wt (g)	Percent ash
Chicken	11.55	71.2	0.62
Beef	11.47	69.9	0.70
Pork	11.41	52.0	0.46
Ham	11.10	220.6	2.0

Samples of the ashed meats and comparators were then prepared and packaged for irradiation. Two-gram portions of each ash were packed in aluminum-foil cylinders. These cylinders were tightly crimped and sealed in quartz ampoules.

Three primary comparator samples were prepared for each element, along with secondary comparators for their respective interfering elements. The comparators were also doubly-contained in aluminum foil and quartz. The quartz ampoules were inserted in an aluminum cylinder for irradiation in the Brookhaven Graphite Reactor.

Analytical procedures. Two irradiation experiments were performed. Samples from the first irradiation were used for qualitative analysis for some rare earths and platinum metals. Quantitative determinations of 19 additional elements were made on samples from the second irradiation.

After each irradiation, the ash and comparator samples were dissolved. Aliquots of the solutions were taken for radiochemical separation and purification of the respective activation products, and for measurement of their radiations.

The ash samples were normally dissolved in boiling aqua regia. Any insoluble residue was dissolved with hydrofluoric acid. Metallic comparators were dissolved in an appropriate acid. Closed systems were used to dissolve comparators for volatile elements and ash samples in which chlorine analyses were to be performed.

Known aliquots of each solution were taken for the specific elemental analyses. Since only tracer quantities of the activation products are produced in such irradiations, known quantities of each element were added to the respective aliquots to serve as carriers. The carriers facilitate chemical separations and also indicate the fractional recovery, or chemical yield, at the completion of the purification procedure, upon analysis by conventional techniques.

Each activation product was identified by measurement of its half-life and the energies of its beta or gamma radiations. The energies of gamma radiations were measured with a flat 3×3-in. NaI(Tl) crystal and a 256-channel pulse-height analyzer. End-window, methane-flow proportional counters were used to measure beta radiations. Beta energies were derived from aluminum absorption curves. As an additional check of sample purity, the decay of each sample was followed for several half-lives, when possible, to verify the half-life.

The procedures for the qualitative analyses involved group separations of the rare earths and platinum metals, followed by separations within each group. The separations of the platinum metals were based on the system described by Noyes and Bray (1943). The rare earths were separated into the yttrium and lanthanum groups by successive extractions of the yttrium group into tributyl phosphate. Many of the quantitative analysis procedures were modifications of standard radiochemistry procedures (Kleinberg, 1958; Meinke, 1949). For some elements, essentially new procedures were developed.

EXPERIMENTAL RESULTS

Qualitative analyses. The presence of several platinum metals and of several rare earths in meat ash was established by qualitative neutron activation analysis.

The radionuclides 40-day Ru^{103}, 17-day Pd^{103}, 74-day Ir^{192}, and 3.4-day Pt^{193m} were identified by the energies of their beta or gamma radiations and their half-lives. Estimates were made of the order of magnitude of the concentrations of these elements in each food sample. The values, which range from $\sim 10^{-4}$ to 10^{-6} ppm in the bulk meats, are shown in Table 4.

Certain rare earth elements were identified from sequential gamma spectrum analyses of the yttrium and lanthanum groups. Typical gamma spectra of the yttrium fraction from the beef ash sample are presented in Fig. 2. Spectrum A was taken approximately two months after the irradiation, and spectrum B was taken after an additional two-month decay. Spectra are essentially the same for the yttrium fractions from the other meat ash samples. The most prominent photopeaks appear in channels 89, 110, and 200. These peaks correspond to gamma energies of 0.88 Mev, 1.12 Mev, and 2.0 Mev, and arise from the decay of 85-day Sc^{46}. Although scandium follows yttrium group chemistry, its rather high concentration in these samples was unexpected. Therefore, a quantitative analysis for scandium was performed in a subsequent irradiation.

Because of the comparatively large amount of scandium activity in these spectra, the sensitivity for the detection of other constituents was decreased substantially. However, it was possible to identify photopeaks due to the 84-Kev photon of 129-day Tm^{170} and to several photons of 32-day Yb^{169} and 6.8-day Lu^{177}.

Gamma spectra for the lanthanum fraction from the beef ash sample are presented in Fig. 3. These spectra were taken at the same time as those for the yttrium fraction. Spectra for the other ash samples were nearly identical. The most prominent photopeaks, corresponding to photon energies of 0.120 Mev, 0.34 Mev, 0.86 Mev, and 1.09 Mev, indicate the presence of 13-year Eu^{152} and 16-year Eu^{154}. There is also some evidence for a 20-Kev photon, which may indicate the presence of 80-year Sm^{151}.

Estimates were made of the concentrations of these rare earth elements in each meat. The values, as shown in Table 4, are of the order of 10^{-6} to 10^{-5} ppm.

Quantitative analyses. The concentrations of 19 elements in each meat ash were determined in samples from the second irradiation. The analytical data are shown in Table 5, along with the concentrations in the bulk meats, which were cal-

Table 4. Concentrations (ppm) of trace elements in meat (qualitative analyses).

Trace element	Chicken		Beef		Pork		Ham	
	Ash	Bulk	Ash	Bulk	Ash	Bulk	Ash	Bulk
Ruthenium	0.1	6×10^{-4}	0.1	7×10^{-4}	10^{-2}	5×10^{-4}	0.1	2×10^{-3}
Palladium	0.1	6×10^{-4}	0.1	7×10^{-4}	0.1	5×10^{-4}	0.1	2×10^{-3}
Iridium	10^{-3}	6×10^{-6}	10^{-3}	7×10^{-6}	10^{-3}	5×10^{-6}	10^{-3}	2×10^{-6}
Platinum	0.1	6×10^{-4}	0.1	7×10^{-4}	0.1	5×10^{-4}	0.1	2×10^{-3}
Ytterbium	10^{-2}	6×10^{-5}	10^{-2}	7×10^{-5}	10^{-3}	5×10^{-6}	10^{-3}	2×10^{-5}
Thulium	10^{-2}	6×10^{-6}	10^{-3}	7×10^{-6}	10^{-3}	5×10^{-6}	10^{-3}	2×10^{-5}
Lutetium	10^{-2}	6×10^{-5}	10^{-2}	7×10^{-5}	10^{-2}	5×10^{-3}	10^{-3}	2×10^{-3}
Europium	10^{-2}	6×10^{-5}	10^{-2}	7×10^{-5}	10^{-3}	5×10^{-6}	10^{-3}	2×10^{-3}

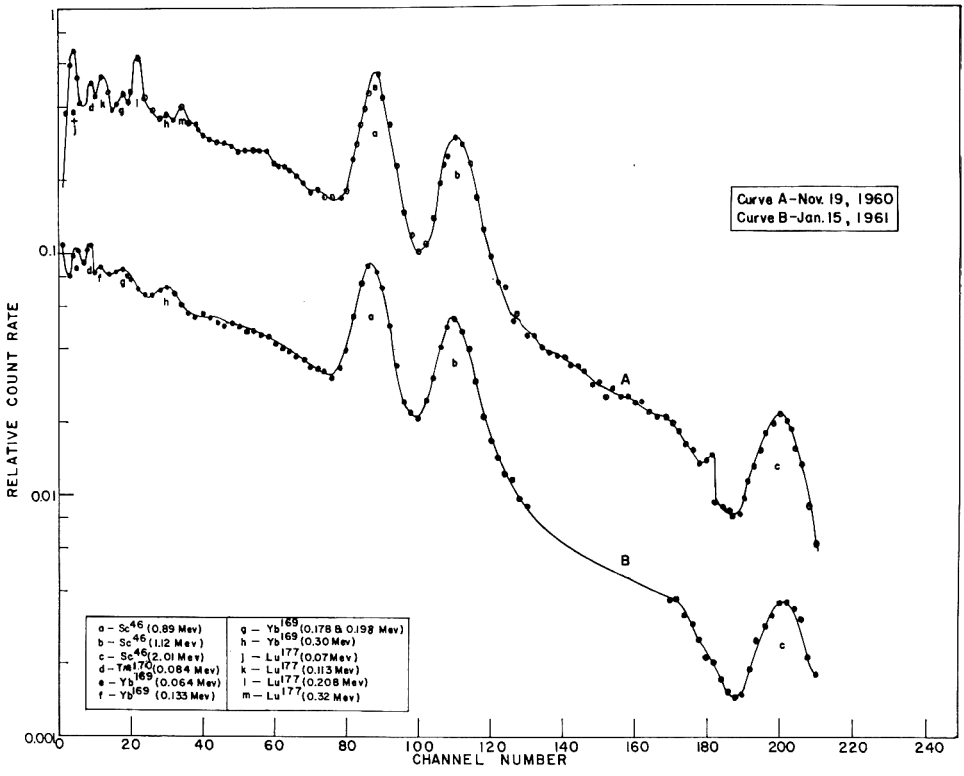

Fig. 2. Gamma spectra for yttrium group in irradiated beef ash.

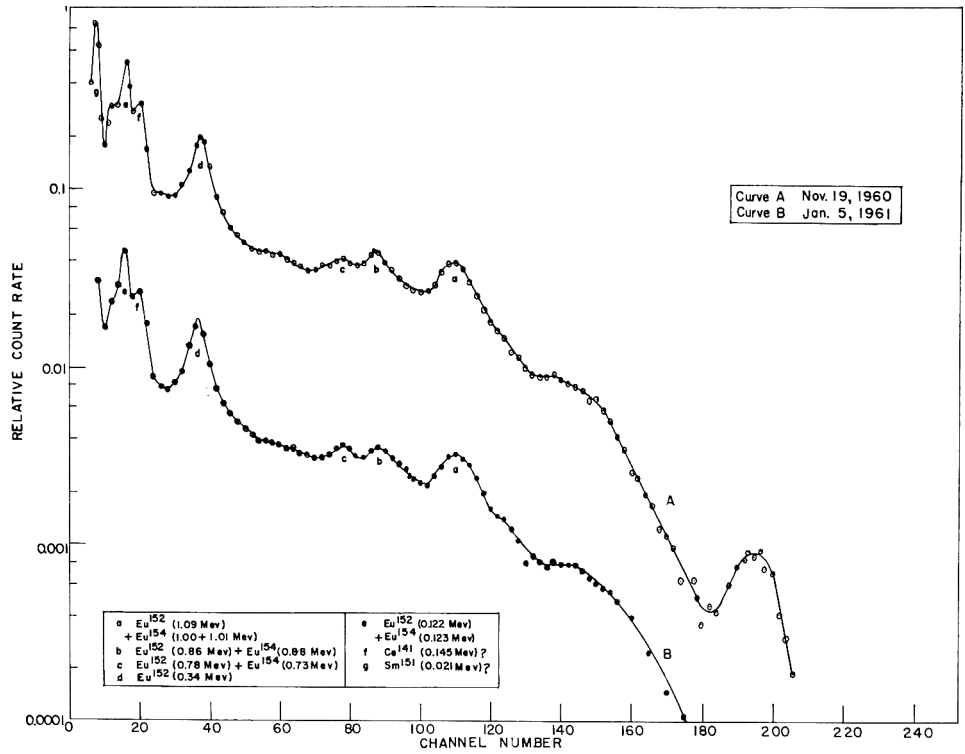

Fig. 3. Gamma spectra for lanthanum group in irradiated beef ash.

Table 5. Concentrations (ppm)[a] of trace elements in meat (quantitative analyses).

Element	Chicken		Beef		Pork		Ham	
	Ash	Bulk	Ash	Bulk	Ash	Bulk	Ash	Bulk
Phosphorus	2.0×10^5	1.2×10^3	1.9×10^5	1.3×10^3	2.0×10^5	9.2×10^2	5.2×10^4	1.0×10^3
Sulfur	$<2 \times 10^4$	$<1 \times 10^2$	$<8 \times 10^4$	$<6 \times 10^2$	$<4 \times 10^4$	$<2 \times 10^2$	$<5 \times 10^5$	$<1 \times 10^4$
Chlorine	3.2×10^3	....	1.8×10^4	....	9.2×10^3	....	9.9×10^4	....
Scandium	1.2	7.4×10^{-3}	0.79	5.5×10^{-3}	0.57	2.6×10^{-3}	0.12	2.4×10^{-3}
Iron	2.1×10^4	1.3×10^2	5.8×10^3	41	4.2×10^4	19	3.9×10^3	78
Cobalt	17	0.11	4.5	3.2×10^{-2}	4.0	1.8×10^{-2}	3.0	5.9×10^{-2}
Zinc	1.7×10^3	11	2.9×10^3	20	3.0×10^3	14	4.8×10^2	9.5
Selenium	6.6×10^{-2}	4.1×10^{-4}	3.9×10^{-2}	2.7×10^{-4}	1.7×10^{-2}	7.8×10^{-5}	1.3×10^{-2}	2.6×10^{-4}
Rubidium	3.9×10^3	24	1.8×10^2	1.3	2.8×10^2	1.3	7.5×10^2	15
Strontium	35	0.22	25	0.18	20	9.2×10^{-2}	19	0.38
Zirconium	<16	<0.10	<24	<0.2	<30	<0.1	<5	<0.1
Indium	0.75	4.7×10^{-3}	1.3	9.2×10^{-3}	0.47	2.2×10^{-3}	0.55	1.1×10^{-2}
Tin	17	0.11	77	0.54	14	6.4×10^{-2}	3.2	6.4×10^{-2}
Tellurium	1.0 ± 0.5	$(6 \pm 3) \times 10^{-3}$	0.7 ± 0.14	$(5 \pm 1) \times 10^{-3}$	0.7 ± 0.14	$(3 \pm 0.6) \times 10^{-3}$	1.0 ± 0.2	$(2.0 \pm 0.4) \times 10^{-2}$
Cesium	1.7	1.1×10^{-2}	1.3	9.2×10^{-3}	1.3	6.0×10^{-3}	0.33	6.6×10^{-3}
Barium	53	0.33	50	0.35	38	0.18	19	0.38
Cerium	$(1.7 \pm 0.4) \times 10^{-3}$	$(1.1 \pm 0.3) \times 10^{-5}$	$(5.4 \pm 1.4) \times 10^{-4}$	$(3.8 \pm 1.0) \times 10^{-6}$	$(3.9 \pm 1.0) \times 10^{-3}$	$(1.8 \pm 0.4) \times 10^{-5}$	$(7.5 \pm 1.9) \times 10^{-4}$	$(1.5 \pm 0.4) \times 10^{-5}$
Hafnium	3.1	1.9×10^{-2}	3.9	2.0×10^{-2}	4.2	1.9×10^{-2}	0.60	1.2×10^{-2}
Uranium	1.1 ± 0.2	$(6.8 \pm 1.0) \times 10^{-3}$	0.50 ± 0.07	$(3.5 \pm 0.5) \times 10^{-3}$	0.48 ± 0.07	$(3.4 \pm 0.5) \times 10^{-3}$	0.19 ± 0.03	$(3.8 \pm 0.6) \times 10^{-3}$

[a] Errors are estimated to be less than or equal to $\pm 10\%$ except where indicated.

culated from the ratios of ash to bulk weights given in Table 3. Definitive values were obtained for all of the elements listed except zirconium and sulfur.

Zirconium was determined in a purified mixture of zirconium and hafnium by gamma spectrometry techniques. However, the hafnium photo-peaks in the spectrum were sufficiently predominant to mask those of Zr^{95}. The values of the upper limits for zirconium concentrations were calculated from estimates of the minimum quantity of Zr^{95} that could be detected in the presence of the hafnium activity in each sample. The limiting values for zirconium concentrations are large relative to the observed hafnium concentrations because the cross-section of zirconium for thermal neutron activation is much smaller than that of hafnium. If more definitive data for zirconium concentrations had been desired, specific chemical separations could have been performed to permit measurement of the radioactivity due to zirconium.

The special problems associated with the determination of phosphorus and sulfur are discussed in detail in Appendix I. The very large upper limits for the concentrations of sulfur are indicative of the serious interference due to the $Cl^{35}(n,p)S^{35}$ reaction in chlorine. The reported value of the upper limit is the sulfur concentration, which corresponds to the minimum quantity of sulfur-produced S^{35} that is detectable in the presence of the chlorine-produced S^{35} in each sample. For chicken, pork, and beef, the total S^{35} found was identical, within experimental error, to the chlorine-produced S^{35}. In the case of ham ash, the minimum detectable quantity of sulfur was unrealistically high. Therefore, for each case, it is concluded that chlorine interference precludes determination of sulfur in these samples by neutron activation analysis.

In general, the value of the sulfur concentration is a prerequisite to determination of phosphorus, to permit estimation of the effects due to the reaction $S^{32}(n,p)P^{32}$. However, the observed phosphorus concentrations and the concentrations of P^{32} in the sulfur comparators were used to estimate the sulfur concentration that would have been required to introduce a 10% error in each phosphorus analysis. The estimated sulfur concentration was 63% in ham ash and over 100% for the other ashes. Therefore, the maximum error introduced in the phosphorus analyses by neglecting the effects of sulfur interference was of the order of a few percent.

The interference due to fast neutron-induced reactions in analysis for other elements was also determined. However, for all experimentally determined elements, correction factors were negligibly small. Therefore, no significant errors were introduced by these effects.

In general, precisions of replicate determinations of elemental concentrations were maintained to better than $\pm 5\%$. On the basis of these precisions and of the evaluation of possible consistent errors in sample preparation and analysis, it is estimated that the values of the concentrations of most of the trace elements were determined with an accuracy of $\pm 10\%$. All results in Table 5 are judged to have this accuracy except where larger errors are given.

DISCUSSION

The analyses yielded definite values for the concentrations of seventeen of the elements. Their concentrations in the bulk meats ranged from values of $\sim 1{,}000$ ppm, for phosphorus, to $\sim 10^{-6}$ ppm, for cerium. Interferences precluded the determination of the concentrations of two elements, sulfur and zirconium. Therefore, maximum values for their concentrations, based on the limits of sensitivity of the methods employed, are reported.

Qualitative neutron activation analyses resulted in detection of four platinum metals and four rare earth elements. Estimated values for the concentrations of these elements range from $\sim 10^{-3}$ ppm to $\sim 10^{-6}$ ppm in the bulk meats.

The reported concentrations for chlorine in the ash samples are considered accurate to $\pm 10\%$. However, calculation of chlorine concentrations in the bulk meats from the ash analyses is not deemed valid, because of possible loss of chlorine through volatilization during the ashing operation. Direct analysis of the bulk meat for chlorine is probably required to determine its fractional loss, if any, during ashing. Therefore, no value is reported for chlorine concentration in the bulk meats.

The extent to which the concentration of an individual element is constant in the four meats is indicated by equation 3:

$$\Delta c = \frac{\text{maximum observed concentration of an element in a meat}}{\text{minimum observed concentration of an element in a meat}} \quad [3]$$

The distribution of the elements for varying values of Δc is summarized in Table

Table 6. Variations of elemental concentrations in ashed and bulk meat.

Concentration variation (Δ c)	Number of elements	
	Ashed meat	Bulk meat
$\Delta c < 2$	2	6
$2 < \Delta c < 4$	2	3
$4 < \Delta c < 8$	9	5
$8 < \Delta c$	4	2
Total	17[a]	16[a]

[a] Chlorine data for ash only.

6. The values of the respective concentrations of a majority of the elements in the four meats were constant within a factor of eight. Furthermore, the concentrations of 9 elements were consistent within a factor of four in the bulk meats, and in the ash samples the range of 13 elemental concentrations is greater than a factor of four. These variations seem to indicate that the concentrations of the majority of these elements in these meat samples are related to the organic content of the samples and do not maintain a constant relationship with the principal inorganic constituents.

In view of the significant variations in elemental concentrations observed in different samples of the same food (Winton and Winton, 1949), it does not appear valid to assume that the concentration data for these samples typify the four meats. However, the results may be indicative of the order of magnitude of the concentration of each element.

APPENDIX

Activation Analysis for Phosphorus and Sulfur

Neutron activation analysis for phosphorus, using the activation reaction $P^{31}(n,\gamma)P^{32}$, is complicated by the possible interference of sulfur and/or chlorine in the matrix sample from the reactions $S^{32}(n,p)P^{32}$ and $Cl^{35}(n,\alpha)P^{32}$ as indicated in Fig. 1. Similarly, activation analysis for sulfur, using the reaction $S^{34}(n,\gamma)S^{35}$, is subject to interference from the reaction $Cl^{35}(n,p)S^{35}$. Since 14.2-day P^{32} is the only neutron activation product available for phosphorus, and 87-day S^{35} is the only sulfur activation product with a half-life of useful magnitude for many applications, neutron activation analysis techniques for these two elements must take into account the need for corrections in the analytical data for the effects of the interfering elements.

Since the total quantity of S^{35} produced during the irradiation of the matrix is the sum of the contributions from the $S^{34}(n,\gamma)S^{35}$ and $Cl^{35}(n,p)S^{35}$ reactions, it is necessary to differentiate between the S^{35} produced by these two reactions. This can be accomplished by measuring the total S^{35} content of the matrix and the S^{35} produced from the chlorine in the matrix. The S^{35} produced from the sulfur in the matrix is the difference between these two quantities.

To make these measurements, the concentration of chlorine in the matrix must be determined by some method. Activation analysis, using the reaction $Cl^{35}(n,\gamma)Cl^{36}$, was the method chosen for this program.

In a similar manner, in phosphorus analyses, the total quantity of P^{32} must be determined along with the quantities produced by the $S^{32}(n,p)P^{32}$ and the $Cl^{35}(n,\alpha)P^{32}$ reactions. The amount of P^{32} due to activation of phosphorus is the balance remaining after subtraction of the contributions of the two interfering reactions from the total P^{32} found. Therefore, the concentrations of both sulfur and chlorine must be known in order to compute the concentration of phosphorus. The methods used for these determinations are detailed below.

Table 7. Method of analysis for sulfur.

Data required	Experimental operation
1. Cl concentration in ash (μg Cl/g ash)	1a Determine Cl^{36} in ash
	1b Determine Cl^{36} in Cl comparator
	1c Calculate Cl concentration
2. S^{35} production from Cl (dpm S^{35}/μg Cl)	2a Determine S^{35} in Cl comparator
3. S^{35} production from Cl in ash (dpm S^{35} (Cl)/g ash)	3a Calculate: item (2) $\times$ item (1)
4. Total S^{35} production in ash (dpm S^{35}/g ash)	4a Determine S^{35} in ash
5. S^{35} production from S in ash (dpm S^{35} (S)/g ash)	5a Calculate: item (4) $-$ item (3)
6. S^{35} production from S (dpm S^{35}/μg S)	6a Determine S^{35} in sulfur comparator
7. S concentration in ash (μg S/g ash)	7a Calculate: item (5) $\div$ item (6)

Table 8. Method of analysis for phosphorus.

Data required		Experimental operations	
1.	Cl concentration in ash (μg Cl/g ash)	1a	See operation no. 1c, Table 7
2.	S concentration in ash (μg S/g ash)	2a	See operation no. 7a, Table 7
3.	P^{32} production from Cl (dpm P^{32}/μg Cl)	3a	Determine P^{32} in Cl comparator
4.	P^{32} production from Cl in ash (dpm P^{32} (Cl)/g ash)	4a	Calculate: item (3) $\times$ item (1)
5.	P^{32} production from S (dpm P^{32}/μg S)	5a	Determine P^{32} in S comparator
6.	P^{32} production from S in ash (dpm P^{32}(S)/g ash)	6a	Calculate: item (5) $\times$ item (2)
7.	Total P^{32} production in ash (dpm P^{32}/g ash)	7a	Determine P^{32} in ash
8.	P^{32} production from P in ash (dpm P^{32}(P)/g ash)	8a	Calculate: item (7) $-$ [item (6) $+$ item (4)]
9.	P^{32} production from P (dpm P^{32}/μg P)	9a	Determine P^{32} in P comparator
10.	P concentration in ash (μg P/g ash)	10a	Calculate: item (8) $\div$ item (9)

SULFUR ANALYSES

Sulfur analyses require the irradiation of comparator samples for both sulfur and chlorine along with the ash samples. The data required and the method of obtaining them are shown in Table 7.

If the ratio of the concentration of chlorine to sulfur in the ash is relatively large, the calculation performed in Operation No. 5a in Table 7 may result in a small difference term of two large numbers. The smallest statistically significant value of this difference represents the minimum quantity of sulfur-produced S^{35}/gram ash that is detectable in the sample. In this case, the calculation in Operation No. 7a yields a value for the upper limit of the sulfur concentration in the ash.

PHOSPHORUS ANALYSES

The analysis for phosphorus requires that comparators for sulfur and chlorine be irradiated along with the matrix and the phosphorus comparators. The data required and the corresponding experimental operations are shown in Table 8.

The extent to which sulfur or chlorine may interfere in the phosphorus analysis is a function of the relative concentrations of the three elements. If the concentration of either sulfur or chlorine is much higher than that of phosphorus, the difference term in Operation No. 8a may be less than the statistical error of the other terms. In this case, analysis for phosphorus would be precluded. The limit of sensitivity for a given sample would then be determined in a manner analogous to that described for sulfur.

A special type of interference due to a second-order neutron reaction may also interfere in analyses for phosphorus. If large quantities of silicon are present, the reaction (Stehn and Clancy, 1956) $Si^{30}(n,\gamma)Si^{31}$ β^- $P^{31}(n,\gamma)P^{32}$ may enhance the production of P^{32} and yield apparent phosphorus concentrations that are too large. However, it was shown (Kruger and Gruverman, 1962) that this reaction was not important in samples containing very high silicon concentrations that were irradiated under similar conditions at the Brookhaven Graphite Reactor. Therefore, it is estimated that this effect was unimportant in these samples.

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Composition of Some Subcellular Fractions from Seeds of Arachis hypogaea [a]

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SUMMARY

Five fractions of parenchymatous cells of the cotyledon of the peanut were isolated by homogenization and differential centrifugation from nonaqueous media. These are two protein-rich fractions (one of which appears to be aleurone grains), starch grains, a fines material, and cell wall fragments. In addition, a fraction composed largely of vascular tissue of the cotyledon was isolated. The nitrogen of the cell is concentrated in the two protein-rich fractions, phytin in the aleurone grains, sucrose mostly in the fines fraction and to a lesser extent in the starch granules, and ribonucleic acid in the fines fraction. The proteins in both protein-rich fractions appear to be the same as judged by chromatography and zone electrophoresis.

The proteins of seeds have been studied generally as fractions of extracts of the entire kernel of specific tissue (Brohult and Sandegren, 1954; Danielson, 1956). Since the major globulins of dicotyledons have no demonstrable biological activity, they have usually been classified as reserve proteins (Danielson, 1956; Steward and Thompson, 1954) and are presently characterized by physicochemical methods. The fractionation of these globulins is complicated by the possibility that the mixing during tissue homogenization in aqueous solutions prior to extraction might produce artifacts caused by protein-protein interaction or by protein interaction with other constituents. One way of obtaining more information about the proteins would be to fractionate the tissue before protein extraction to simplify the source of protein.

Woodroof and Leahy (1940) observed that the settlings from freshly made peanut oil contain aleurone gains, starch grains, and some fragments of cell wall, thus indicating that these structures survive grinding in oil. This fact suggested that subcellular fractionation might be achieved by a variant of the technique of Behrens (1938) and others (Allfrey, 1959) involving homogenization and differential centrifugation in nonaqueous media.

A technique is presented for isolating several fractions of peanut cotyledons: two protein-rich fractions, starch grains, vascular tissue, cell wall fragments, and a fines fraction.

METHODS

Isolation of subcellular fractions. Field-cured NC-2 variety (large-seeded Virginia type) peanuts were stored at 4°C for periods up to one year. A 500-g sample of cotyledons, representing about 250 g of triglyceride-free material, was homogenized in an Omni-Mixer (no endorsement implied), set in an ice bath, and homogenized for

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4 min in 1 L of refined cottonseed oil. After the thick brei was decanted, the residue was homogenized in 1 L of cottonseed oil as before. This process was repeated until most of the cells of the parenchyma were broken. The combined homogenates were centrifuged at 1100 × G for 2.5 minutes; the residue was scrubbed several times by homogenization and centrifugation. This residue was further fractionated by resuspension and centrifugation in media of various densities according to the scheme shown in Fig. 1. Adjustments in density were made with ACS-grade carbon tetrachloride; the various fractions were freed of lipid by extraction with carbon tetrachloride, the last traces of which were removed by vacuum at room temperature.

Analyses. Nitrogen was determined by micro-Kjeldahl (Steyermark, 1951), phosphorus by the technique of Chen *et al.* (1956), moisture by the method of the AOAC (1955), starch polarimetrically according to the method of Clendenning (1945), and phytin according to the technique of Pons *et al.* (1953). Results are reported as the average of duplicate determinations on three different preparations of each subcellular fraction.

Sucrose was determined by the anthrone technique on purified hot 80% ethanol extracts (Snell and Snell, 1953) and identified by partition chromatography on 2% silicic acid paper prepared according to the methods of Dieckert *et al.* (1958) and Dieckert and Reiser (1956). As a further check, the sugar concentrate from the fines material was examined by cellulose paper chromatography (Block *et al.*, 1955).

Ribonucleic acid determinations were made by two methods: first, by absorption of light at 260 mμ of a cold perchloric acid extract prepared by the method of Ogur and Rosen (1950), and second, by absorption of light at 260 mμ of an extract prepared by a modification of the technique of Kirby (1956). The former technique was satisfactory only for the reticulate fraction. The latter system gave more satisfactory results for the other fractions, and agreed satisfactorily

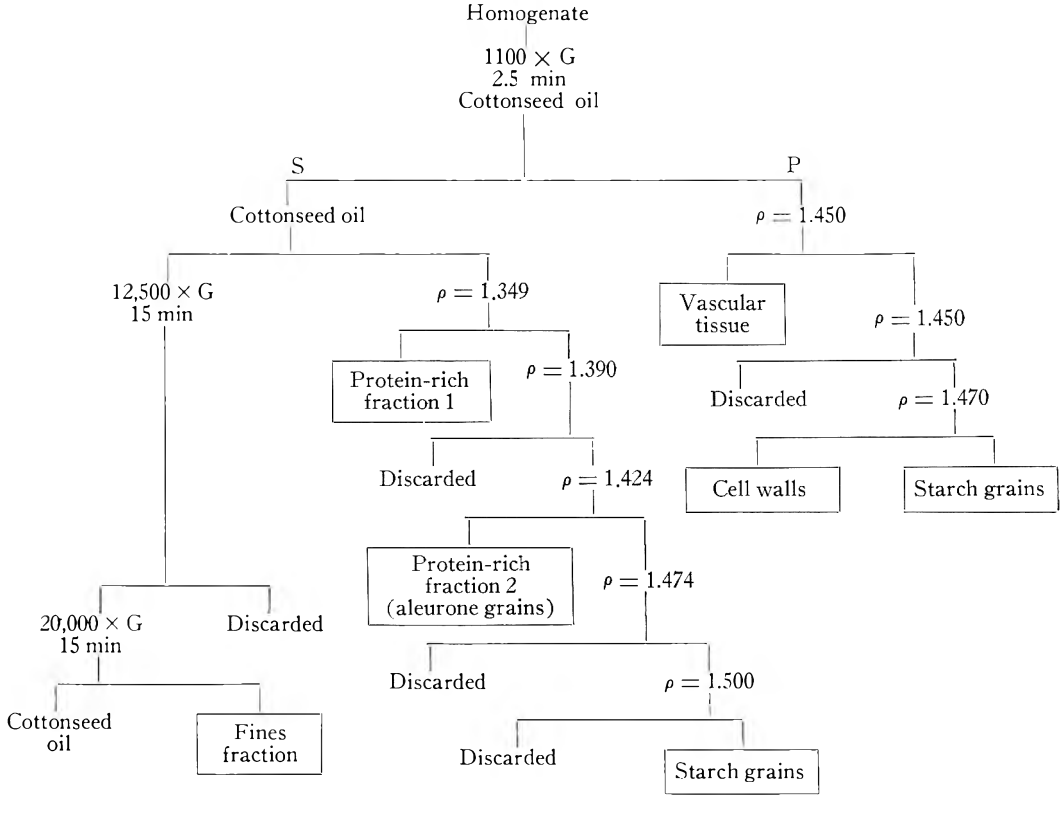

Fig. 1. Scheme for fractionation of peanut cotyledons. S, supernatant; P, precipitate; each step consists of centrifugation after adjusting density; unless otherwise noted, all centrifugations were for 15 min at 2600 × G.

with the results by the Ogur and Rosen technique for the reticulate material.

The modified technique of Kirby was carried out as follows: a 100-mg sample of the material to be analyzed was mixed thoroughly in a conical centrifuge tube with 5 ml of 0.2*M* phosphate buffer, pH 6.8. To the resulting mixture was added 5 ml of 9:10 phenol-water. The two phases that formed were mixed thoroughly and separated by centrifugation. A 3-ml portion of the upper aqueous phase was pipetted into a clean centrifuge tube, and the phenol was removed by repeated extractions with ethyl ether. Residual ether was evaporated under a stream of air at room temperature. Ribonucleic acid was precipitated from solution with 2 volumes of ethanol and collected by centrifugation. After the supernatant liquid was decanted carefully so as not to lose any of the precipitate, the residue was dissolved in a suitable volume of water and read in the spectrophotometer at 260 mμ. An E_p of 9800 (Ogur and Rosen, 1950) and a phosphorus content of 10% were assumed for the ribonucleic acid of peanuts.

Analysis for desoxyribonucleic acid was by two independent techniques: detection of nuclei by counterstaining osmium-fixed sections with aceto-orcein stain (Kurnick and Ris, 1948), and direct assay by the method of Ceriotti (1952).

Calcium, magnesium, and potassium were determined by flame photometry with a Beckman DU spectrophotometer (Bills *et al.*, 1949; Kuemmel and Karl, 1954). Samples of the subcellular particles (100 mg) were treated with 1 ml of 15.4*M* nitric acid and placed in a pre-ashing assembly made according to Thiers (1957). The ashing was completed in a muffle furnace at 450°C.

RESULTS

Visual observations. Intact cells and each of the fractions were characterized in a preliminary way by light and electron microscopy. The storage parenchyma cells are packed with relatively large particles. There is a centrally located nucleus, numerous aleurone grains and translucent spheres, and scattered starch grains. The cell wall and starch grains show a characteristic birefringence between crossed polarizers. Intercellular spaces are prominent.

Electron micrographs of sections of intact cells disclose a three-dimensional network pervading the space between the particles. This reticulate structure is joined to the cell wall and appears to surround the other bodies.

The two protein-rich fractions and the starch-grain fraction were composed of spheres ranging in diameter from 1 to 10 μ. Protein-rich fraction 1 was composed of translucent spheres that showed no birefringence and no inclusions. The spheres of fraction 2 contained one or more spherical inclusions (globoids); these particles corresponded to the classical description of aleurone grains (Esau, 1960; Guilliermond, 1941; Mottier, 1921). The starch grains showed characteristic birefringence between crossed polarizers.

When examined by light microscopy the fines fraction showed few contaminating spherical particles. Preliminary examination by electron microscopy suggests that this fraction was composed of fragments of a network, presumably the network observed in the intact cells.

The vascular-tissue fraction contained relatively large pieces composed of the typical thick-walled, relatively undamaged, cells of the vascular tissue. It was not completely free of components of the parenchyma. The cell wall fraction was relatively free of protein-rich bodies and nuclei, but contained a few starch grains.

Yield and composition of fractions. The nitrogen of the peanut cotyledon was concentrated in the two protein-rich fractions (Table 1). That this nitrogen is largely protein nitrogen was confirmed by its being nondialyzable and yielding amino acids (as determined by paper chromatography) on acid hydrolysis. No substantial portion of this nitrogen could be accounted for as nucleic acid, which was in low concentration in these fractions. Some of the nitrogen of the starch

Table 1. Composition of subcellular fractions.

Fraction	Yield (%)	Moisture (%)	N (%)	P (%)	Starch (%)	Sucrose (%)	Phytic acid %
Protein-rich fraction 1	6.6	9.0	13.3	0.32	0	4.3	0.5
Protein-rich fraction 2 (aleurone grains)	11.6	9.7	11.4	1.87	0	9.5	5.7
Starch grains	3.1	7.9	1.5	0.31	55	17.7	0.5
Fines material	2.0	8.0	6.7	0.71	0	36.0	0.01
Cell wall	1.8	12.9	2.7	0.09	0	2.8	0.01
Vascular tissue	6.0	10.4	7.7	0.78	.....	13.1	0.01
Lipid-free cotyledons		10.0	9.0	0.90	8	9.9	1.7

grains and cell wall fractions might have been associated with contaminating protein-rich particles, the last traces of which are difficult to remove.

Phytin is known to be one of the major forms of phosphorus in the peanut. Protein-rich fraction 2 (aleurone grains) contained the highest concentration of phosphorus, and the bulk of this was phytic acid (Table 1). An appreciable portion of the phosphorus of the other fractions could be accounted for as phytin phosphorus arising from contamination with fraction 2. And this may also be true for even the low phosphorus content of the cell wall. The aleurone grains were highest in potassium and magnesium content (Table 2); presumably some of this is bound with phytic acid as mixed potassium and magnesium salts.

Table 2. Distribution of some metals in subcellular fractions.

Fraction	Ash (%)	Ca (%)	K (%)	Mg (%)
Protein-rich fraction 1	4.68	0.03	1.38	0.09
Protein-rich fraction 2 (aleurone grains)	11.07	0.04	2.78	0.73
Starch grains	2.63	0.01	0.63	0.09
Fines material	3.37	0.01	0.97	0.06
Cell wall	2.87	0.35	0.54	0.34
Lipid-free cotyledons	6.44	0.06	1.66	0.38

Sucrose had a striking intracellular distribution. The fines fraction contained 36% sucrose. The sucrose content of the aleurone grains was about twice that in protein-rich fraction 1, but only about one-fourth that in the fines fraction. Starch grains contained 17% sucrose. The cell walls contained only 2.8%, and this may have been associated with contaminating fines material.

Nucleic acid was localized in the fines fraction (Table 3); the ultraviolet spectrum confirmed that the absorption at 260 mμ was of nucleotides. Desoxyribonucleic acid was not present in this fraction; it could not be found by chemical analysis and no nuclei could be detected by staining. Most of the nucleotide material of this fraction was acid-insoluble as indicated by ribonucleic acid analysis by the method of Ogur and Rosen (1950). The latter gave an estimate of 5.1%, compared with 2% from the modified Kirby technique.

It seems likely that the ribonucleic acid values for the other fractions are somewhat too high since the ratios of light absorption at 260–280 and 260–240 mμ are significantly lower than would be expected on the basis of results with ribonucleic acid from the fines fraction and from yeast.

DISCUSSION

Protein, starch, phytic acid, sucrose, and RNA are clearly concentrated by fractionation in a nonaqueous medium. Calcium is concentrated in the cell walls; potassium and magnesium are concentrated in the aleurone grains. This procedure may be used profitably to isolate constituents that are in low concentration in the entire tissue but that can be concentrated considerably prior to isolation. Such a prior, nonaqueous concentration might avoid changes resulting from general mixing of the seed constituents, and even the production of artifacts. Thirty percent of the original mixture was recovered in the relatively clean fractions.

The two fractions highest in protein differ from each other in density, metal content, and content of phytic acid. However, chromatography on DEAE cellulose by the method of Dechary *et al.* (1961) and zone electrophoresis by the method described by Evans *et al.* (1962) indicate that the proteins in these two fractions are the same, and that these two fractions represent the major sources of protein from the cotyledon. The concentration of phytic acid in one fraction (aleurone grains) provides the opportunity to investigate the interactions between phytic acid and the seed proteins.

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Table 3. Intracellular distribution of ribonucleic acid.

Fraction	% RNA	$\frac{260^a}{240}$	$\frac{260^a}{280}$
Protein-rich fraction 1	0.11	1.61	0.52
Protein-rich fraction 2 (aleurone grains)	0.29	1.31	1.69
Starch grains	0.18	1.81	1.82
Fines material	1.91	2.27	2.05
Cell wall	0.05	1.97	1.94
Vascular tissue	0.50	1.78	1.90
Lipid-free-cotyledons	0.32	1.70	1.81
Yeast RNA	.....	.....	2.23

[a] Ratio of optical density at the wavelength indicated.

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