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Effect of Storage Conditions on Some Physicochemical Properties in Experimental Sausage Prepared from Fibrils [a]

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(Manuscript received January 24, 1961)

SUMMARY

Intact fibrils (a), actin- and tropomyosin-poor fibrils (b), and synthetic fibrils (c) were prepared from beef *M. semitendinosus* within 2 hr of slaughter. Myosin A was prepared from rabbit muscle. The three kinds of fibrils were incubated at 35°C with a trace of toluene. Then the binding quality of sausage prepared from these materials at regular intervals was examined by estimation of elasticity in connection with other physicochemical properties. In (a) and (b), the changes in the binding were in fair agreement with those characteristic of whole muscle sausage made immediately after death. On the other hand, the changes in binding in (c) resembled in many ways those of whole muscle used after storage at 4°C for 7 days after death. Hence, actin and tropomyosin do not greatly influence the binding quality of sausage. It could not be shown that the amount nitrogen soluble in Weber-Edsall solution of 0.6*M* NaCl, or that pH value or water-holding capacity directly influenced the binding quality of the finished products. The course of inactivation of ATPase observed when fibrils were incubated was very similar to that of typical myosin B (in which denaturation takes place in two fairly distinct stages), resembling especially the first stage of the denaturation reaction. From the observation that the first stage of the denaturation of myosin B-ATPase was due to the denaturation of myosin A contained in the preparation, it was concluded that the myosin A present in fibrils is an important substance exerting great influence on the binding quality of sausage.

As reported in a preceding paper (Fukazawa *et al.*, 1961), water-soluble protein had hardly any influence on the binding quality of meat, and there were no differences in binding quality between sausage prepared from whole muscle and sausage prepared from intact fibrils. Though no close relationship was found between whole muscle and isolated myosin A and myosin B, a source of the binding quality of sausage in these studies, further study was recommended to relate the three previous studies (Hashimoto *et al.*, 1959; Yasui *et al.*, 1958, 1960) more closely.

EXPERIMENTAL

Materials. Beef *M. semitendinosus* was used within 2 hours of slaughter. Adenosin triphosphate (ATP) was isolated from fresh rabbit skeletal muscle by Kerr's (1941) method and used as the potassium salt. Myosin A used in the preparation of synthetic fibrils was isolated as described by Perry (1955): after fresh rabbit muscle was extracted with 3 volumes of the Straub solution (0.3*M* KCl, 0.09*M* KH_2PO_4, 0.06*M* K_2HPO_4) for 15 min. at 0°C, the "dilution-precipitation" method was used to isolate pure myosin A. Myosin A, used as a sol, was adjusted to 0.4*M* NaCl in final concentration. For use in the gel stage, myosin A was reduced in ionic strength to less than 0.04 μ by washing a 0.5*M* KCl concentration with 11.5 vol of distilled water.

[a] In this paper, purified actin-free myosin and actin-combined myosin prepared by extracting muscle for 24 hours with 0.6*M* NaCl Weber-Edsall solution are respectively called myosin A and myosin B (natural actomyosin). The term "myosin" is also conventionally used to indicate both the proteins.

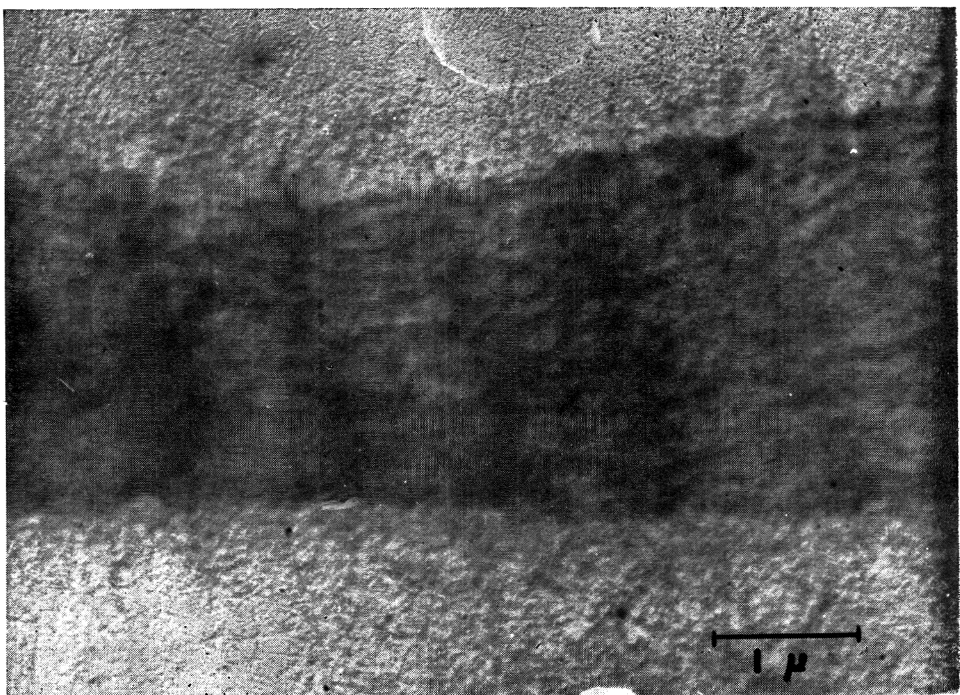

Fig. 1. An electron micrograph of a portion of a synthetic fibril consisting of a "ghost" fibril plus isolated myosin A of rabbit. Myosin A has been extended to all the bands. X16,000.

Intact fibrils, actin- and tropomyosin-poor fibrils, and "ghost" fibrils were prepared as reported in a preceding paper (Fukazawa *et al.*, 1961). Preparation of synthetic fibrils was as follows: 1*M* NaCl solution was added to "ghost" fibrils to a concentration of 0.4*M* NaCl. Protein content in the "ghost" fibrils and in the myosin A was determined beforehand, for estimating the indispensable amount of myosin A that the "ghost" fibrils would require in their structure. It was considered that the amount that must be incorporated was probably 40% of the total protein of "ghost" fibrils. Hence, the myosin A as calculated was poured into the "ghost" fibrils. After the mixture was mixed well, it stood for 24 hr at 0°C: 24 hr later the mixture was washed twice with 10 vol of distilled water and also with 10 vol of 1/40*M* borate-buffer, pH 7.0. Finally the preparation was centrifuged 20 min at 12,000–13,000 rpm at 0°C to obtain a constant moisture content for the uniform condition described in the preceding paper.

Not until 7 days after slaughter were the preparations used as samples.

Procedures. *Electron microscope observation.* Observation by electron microscope was carried out as described in the preceding paper.

ATPase activity. ATPase activity of fibrils and myosin A (in the gel state) was estimated as before (Fukazawa *et al.*, 1961; Yasui *et al.*, 1960), with a suspension in distilled water (protein concentration about 8 mg/ml).

pH values. Fibrils were washed once or twice with 50 vol of various buffers to adjust the pH value, using 1/40*M* acetate buffer (pH 5.7), 1/40*M* tris-maleate buffer (pH 6.4), and 1/40*M* borate buffer (pH 7.05). The pH value of fibrils homogenized with two-fold-distilled water was estimated with a glass electrode pH meter.

Extractable nitrogen and protein determination. The extractable nitrogen with 0.6*M* NaCl Weber-Edsall solution was determined in the following way. About 5 g of the sample fibrils was weighed and suspended with 30 ml of 0.6*M* NaCl Weber-Edsall solution. After standing for 15 min the suspension was centrifuged 15 min at 3000 rpm. The supernatant fluid was filtered through a cloth and the residue was washed several times with the same solution. The total volume of the washing solution was made up to 100 ml, and then the protein count of this solution was determined either by Folin's phenol reagent or by the Biuret method. The protein content, except extractable nitrogen, was estimated by the micro-Kjeldahl method and calculated by multiplying the nitrogen content by a factor of 6.25, or a factor of 6.0 in the case of myosin A.

Sausage manufacturing. The sample sausages were prepared according to the procedures already described (Fukazawa *et al.*, 1961).

Quality of experimental sausage. The quality of the sausage was evaluated by estimating the elasticity and the water-holding capacity of the finished products as described earlier (Hashimoto *et al.*, 1959).

RESULTS

Electron microscopic observation. When structural protein was removed with Hasselbach-Schneider solution (Hasselbach and Schneider, 1951), most of the myosin A and actin was extracted from intact fibrils. The image of the fibrils ("ghost" fibrils) observed by electron microscope at this stage seemed to remain only near the Z-band as reported in the preceding paper; and the appearance, in the present study, of synthetic fibrils, which consisted of "ghost" fibrils taking in myosin A, indicated that the myosin A extended over both the I-band and the A-band, as seen in Fig. 1.

Binding quality of experimental sausage. Sausage prepared from intact fibrils controlled to 5.6, 6.4, and 7.05 pH were used for determining the binding quality. When those sausage materials were incubated at 35°C, changes in elasticity as a function of time were recorded (Fig. 2). Fig. 3 shows the changes in binding quality of sausage made from actin- and tropomyosin-poor fibrils under the same conditions. Fig. 4 shows the elasticity changes in sausage made from synthetic fibrils. It is seen that the binding quality of sausage prepared from intact fibrils decreased generally with time, no matter what the pH value (in the range 5.70–7.05). Temporary recovery of elasticity, however, was observed to occur early in storage, following the same course as the change in binding quality of sausage prepared from whole muscle and incubated at 20°C after storage for 7 days after slaughter at 4°C (Hashimoto *et al.*, 1959). It was concluded that the intact fibrils are similar to the whole muscle with respect to the course of change in binding quality of sausage prepared from fresh material.

Binding quality of sausage made from actin- and tropomyosin-poor fibrils was slightly lower in elasticity value than that of sausage from intact fibrils, but seemed to show the same course of change. In this case, comparisons were carried out at pH 7.0.

As reported earlier (Fukazawa *et al.*, 1961), the sausage made from "ghost" fibrils showed almost no binding quality; but when myosin A was added to such material in the present experiments, and sausage was prepared from the resulting synthetic fibrils, the binding quality of the sausage was increased greatly in comparison with that of the original "ghost" fibrils. The course of change in elasticity of sausage during incubation, as seen in Fig. 4, was identical with that of whole muscle sausage incubated at 20°C immediately after death (Hashimoto *et al.*, 1959).

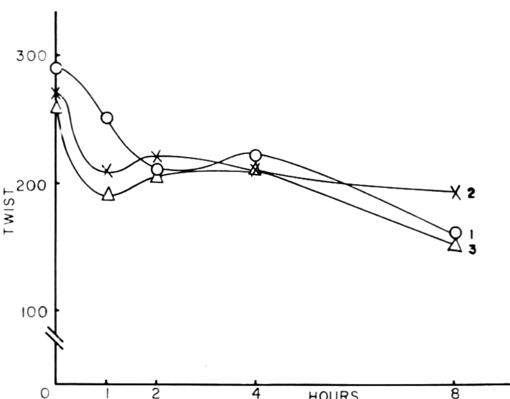

Fig. 2. Change in elasticity of sausage made from intact fibrils stored at 35°C. 1) pH 7.05; 2) pH 6.40; 3) pH 5.70.

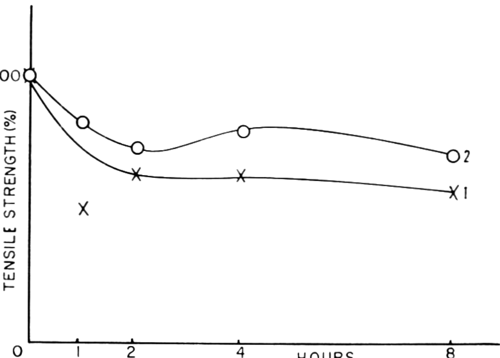

Fig. 3. Change in elasticity of sausage made from actin- and tropomyosin-poor fibrils stored at 35°C. 1) actin- and tropomyosin-poor fibrils; 2) intact fibrils.

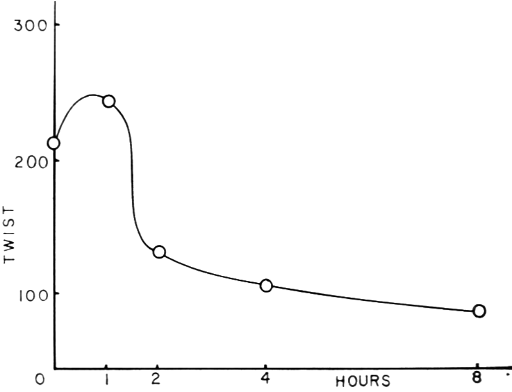

Fig. 4. Change in elasticity of sausage made from synthetic fibrils ("ghost" fibrils + myosin A) stored at 35°C.

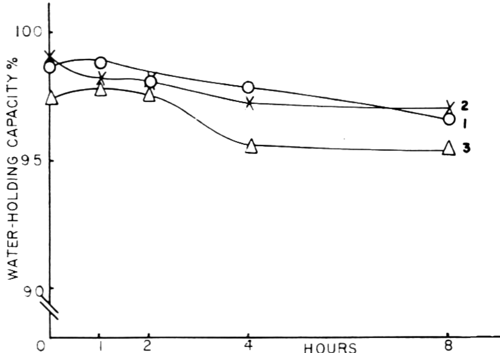

Fig. 5. Change in water-holding capacity of sausage made from intact fibrils stored at 35°C. 1) pH 7.05; 2) pH 6.40; 3) pH 5.70.

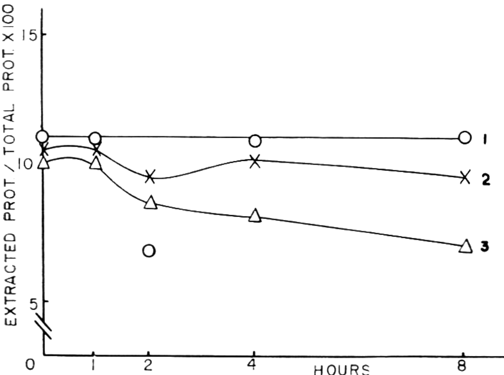

Fig. 6. Change in nitrogen extractable in 0.6*M* NaCl Weber-Edsall solution from intact fibrils from beef *M. semitendinosus* stored at 35°C. 1) pH 7.05; 2) pH 6.40; 3) pH 5.70.

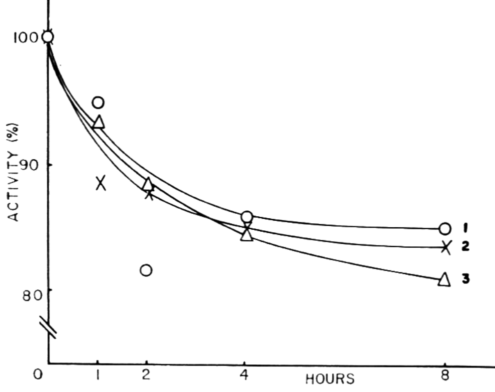

Fig. 7. Change in ATPase activity in intact fibrils stored at 35°C. 1) pH 7.05; 2) pH 6.40; 3) pH 5.70.

Change in water-holding capacity. Studies were carried out on the water-holding capacity of intact fibrils having different pH values. It could not be shown that there was a difference in water-holding capacity between sausage prepared from fibrils at pH 7.05 and that made from fibrils at pH 6.4; but it was clear that the quality of sausage prepared from fibrils at pH 5.6 was lower, as judged by water-holding capacity (Fig. 5). It was thought that the observation described above had yielded adequate results, because the pH value of meat, as is well known, affects the water-holding capacity. However, the water-holding capacity of sausage made from intact fibrils at all three pH values changed during incubation even though the pH of each preparation was always constant throughout the incubation. Therefore, the results suggest that the water-holding capacity of meat might not depend on pH only. For example, there may be an interaction of protein and cations, or a breaking in the stereo structure of the protein molecule, as reported by Hamm and Deatherage (1960). Although the present tests could not solve this problem it was noticed that the binding quality of sausage prepared from intact fibrils is not entirely correlated with the water-holding capacity.

Change in nitrogen extractability. The amount of nitrogen extractable at constant time intervals from intact fibrils with Weber-Edsall solution of 0.6*M* NaCl showed small differences related to the pH value of the sample, as indicated in Fig. 6. The fibrils whose pH was near the neutral region (curve 1) yielded only slightly more nitrogen than fibrils having lower pH values (curves 2, 3). The difference in extractability due to pH value was regarded as reasonable, because hydration of protein usually increases at the neutral region and, in proportion to the increase in hydration, the solubility becomes higher. Such a finding indicated definitely that there was no close relation between the amount of extractable protein and the binding quality of sausage prepared from intact fibrils.

Change in enzymic activity. Changes in ATPase activity of intact fibrils, as shown in Fig. 7, were similar to those observed in experiments on the denaturation of isolated myosin B, as previously reported (Yasui *et al.*, 1958). Such denaturation consisted of a rapid-order reaction independent of pH, and a slow one greatly influenced by the pH value. These facts have led to the conclusion that the components of intact fibrils include myosin-B-like protein. From Figs. 2 and 7 it seems reasonable to conclude that the decrease in binding quality of experimental sausage prepared from intact fibrils corresponds with the inactivation of ATPase in that material. However, as seen in Fig. 2, recovery of the binding quality was observed after 2–4 hours of incubation. Although this phenomenon cannot be

explained in terms of ATPase activity, it appears to be due to the structural decomposition of fibrils, as noted in the case of the recovery of the binding quality of whole-muscle sausage during storage (Hashimoto *et al.*, 1959). Date comparing courses of inactivation of ATPase in both intact fibrils and actin- and tropomyosin-poor fibrils can be seen in Fig. 8, which indicates that both sorts of fibrils were

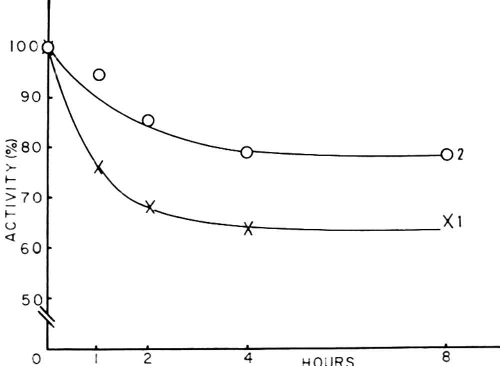

Fig. 8. Change in ATPase activity in actin- and tropomyosin-poor fibrils stored at 35°C. 1) actin- and tropomyosin-poor fibrils; 2) intact fibrils.

similar in this respect. This decrease of ATPase activity in actin- and tropomyosin-poor fibrils may have been due to an increase in free myosin, which did not bind with the actin in the fibrils concomitant with the removal of actin. Considering the data graphed in Figs. 3 and 8, the decrease in binding quality was related to the inactivation of ATPase. The course of decrease in ATPase activity of "ghost" fibrils at regular intervals, as shown in Fig. 9, was like that of myosin B (Yasui *et al.*, 1958); slight ATPase activity was present at the end of incubation. The ATPase activity of myosin A in the gel state was greatly decreased after storage of the material for 8 hr at 35°C. Thereupon, observations were carried out on synthetic fibrils composed of "ghost" fibrils and myosin A. The results obtained show that when the synthetic fibrils were incubated, the time-course of their change in ATPase activity was similar to that of myosin B. It was thought, from this observation, that the remaining actin of "ghost" fibrils combined with myosin A, and that actomyosin, which is comparatively stable to heat, was formed from the two.

DISCUSSION

The studies suggest that the changes in the properties of myosin B were similar to those described previously (Hashimoto *et al.*, 1959; Yasui *et al.*, 1958) on the denaturation of isolated myosin B and muscle homogenate. The relationship between myosin B and muscle homogenate will be more clearly elucidated by studies on the denaturation of protein in fibrils simplified as much as possible. When intact fibrils were observed as experimental sausage, the course of change in the binding quality was the same, during incubation, as that shown by sausage prepared from whole muscle stored after slaughter for 7 days at 4°C, including the transient recovery of binding quality that occurred during incubation. In view of the change in ATPase activity, the suspension of fibrils was seen to be similar to isolated myosin B. Furthermore, the course of decrease in the binding quality of experimental sausage prepared from intact fibrils was nearly identical with the course of inactivation of ATPase of intact fibrils in suspension. It could not be concluded that pH value, water-holding capacity, or nitrogen extractability had any direct influence on the binding quality of sausage prepared from intact fibrils.

In view of these results, the myosin B in fibrils, indeed, should be considered to play an important role in the binding quality of sausage. Actomyosin, the main component of structural protein in muscle, is constituted of actin and myosin in some certain ratio, as well known. From the changing of the ATPase activity shown when the actin was removed from fibrils, it was confirmed that

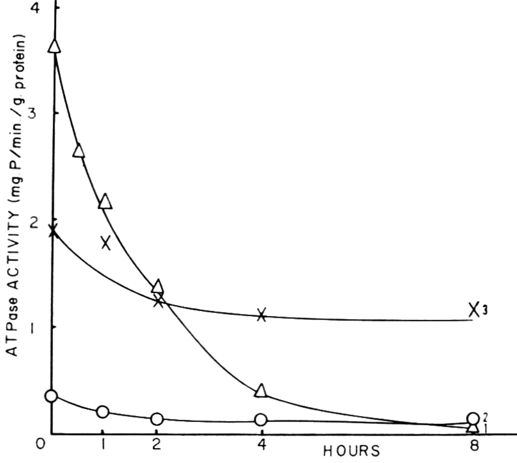

Fig. 9. Change in ATPase activity in rabbit myosin A, "ghost" fibrils and synthetic fibrils stored at 35°C. 1) rabbit myosin A; 2) "ghost" fibrils; 3) synthetic fibrils.

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free-myosin increased so that the so-called myofibrillar protein, in which actin is one component, was present in the fibrils in the form of actomyosin, a combination of actin and myosin. When sausage prepared from actin- and tropomyosin-poor fibrils was compared with sausage made from intact fibrils, not much difference could be observed in changes in binding quality resulting from incubation. Therefore, the actin contained in the fibrils had little effect on the binding quality of the sausage, and must have been present as actomyosin, which is comparatively resistant to heat denaturation. Sausage made from "ghost" fibrils prepared with Hasselbach-Schneider's solution, had very low binding quality, but when rabbit myosin A was added to the "ghost" fibrils, the binding was largely restored. The course of change in the binding quality during incubation seemed similar to that of whole muscle material incubated immediately after slaughter.

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Bitterness in Celery[a, b]

HUO-PING PAN

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(Manuscript received February 1, 1961)

SUMMARY

The bitter principle of celery was isolated by successive solvent extraction, paper chromatography and curtain electrophoresis. It is a colorless crystalline non-flavonoid D-glucoside with mp of 159°C. It is cationic at pH greater than 7, soluble in polar solvents, and fluorescent under ultraviolet light. Electrophoresis is introduced as a new technique in the isolation of a bitter principle from a vegetable. A new conception of bitterness in plants is introduced.

Bitterness in celery (*Apium graveolens* Linn), objectionable to consumers, has long been of concern to vegetable growers, distributors, and processors. The bitterness has been described (Truscott, 1954) and evaluated organoleptically (Hall, 1957), but the elusive bitter principle has not been isolated and studied, even though such basic knowledge is essential for its quantitative evaluation and eventual control. This paper describes isolation of the bitter principle by successive solvent extractions, paper chromatography, and curtain electrophoresis.

The major difficulties encountered were: 1) The bitter principle is present in celery in only a minute quantity; the interfering factors, which would otherwise be negligible, are predominant. 2) The major constituents of celery greatly mask organoleptic detection of the bitter principle in extracts. 3) Celery contains many compounds that fluoresce under UV-L (ultraviolet light of long wavelengths), so paper chromatograms of celery extracts were swamped with fluorescent compounds. 4) No indicator has been found that will react specifically with the bitter principle. 5) The bitter principle does not crystallize when a trace of impurity is present.

Although none of the established procedures for isolating bitter principles from other plants would completely overcome the difficulties encountered with celery, it was found that bitterness appeared to be associated with fluorescence under UV-L, and the bitter principle could be isolated through a tracking procedure with the use of organoleptic sampling technique and infrared absorption spectrum analysis.

EXPERIMENTAL AND RESULTS

Organoleptic sampling was conducted by a taste panel consisting of three regular members. Taste testing was performed in such a way that a minimum amount of isolated material was consumed. Bitter fraction was dissolved in 5 drops of redistilled water delivered from a 1-mm ID capillary. The tasting of the pure bitter principle was conducted in a similar manner, but the amount of water was reduced to about 0.5 drop. The solution was introduced into a capillary, and applied directly to the tongue of the taster with gentle suction.

In the preliminary study, outer petioles of a fresh, over-mature type of celery (variety Utah 52-70) grown in the Belle Glade area of Florida were chopped into small pieces, dehydrated at 60°C to 6.4% dry weight, and ground to 40 mesh in a Wiley mill. A small amount of celery powder was extracted with an organic solvent in a separatory funnel. Petroleum ether, carbon tetrachloride, chloroform of the technical grade, anhydrous and water-saturated diethyl ether, methanol, and ethanol were used individually. Each extract was evaporated to dryness and the residue extracted with a small amount of water. The use

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[a] Presented in part at the Nineteenth Annual Meeting of the Institute of Food Technologists, Philadelphia, Pennsylvania, May 18, 1959.

[b] Florida Agricultural Experiment Station Journal Series, No. 1085.

of water was based on the assumption that the bitter principle must be soluble in water in order to reach the taste buds of bitterness. The water extract was tasted. It was found that the water extract of residue obtained from technical-grade chloroform extract gave a faint, but closer to a pure, bitter taste. Bitterness remained after water was removed from the extract, either by evaporating to dryness at 40°C in an oven with forced draft or by lyophilizing. Direct water extraction was also conducted, but the extract was very impure in bitter taste.

Solvents and solvent-mixtures were investigated for isolation of the bitterness on paper strip in the tube designed by Pan (1956). Water, BAW (*n*-butanol–acetic acid–water, 4:1:5), and 2% acetic acid were found to separate the residue, obtained from lyophilization as well as from evaporation, into spots that fluoresced under UV-L. With water as solvent the spot closest to the front of R_f 0.85 was diffused and not well defined. It was yellow under VL (visible light), and bluish yellow in fluorescence under UV-L. A similar spot, closest to the front, was noticed on the strip when BAW or 2% acetic acid was used as solvent. No fluorescent spot was noticed on the strips under UV light of short wavelengths.

Descending paper chromatograms on 4 × 22½-in. sheets of paper were prepared with water, BAW, and 2% acetic acid as solvents. More narrow fluorescent bands appeared.

The bitter zone on a paper chromatogram was located entirely by tasting, since no indicator has been found that will react specifically with the bitter principle. The edges of the fluorescent bands served, however, as convenient arbitrary guiding lines in cutting the whole chromatograms into fractions. Each fraction was extracted with methanol under reflux. After removal of alcohol by evaporation, the material extracted from each fraction of the chromatograms was dissolved in water and tasted.

The bitter zone on the chromatograms with water, BAW, and 2% acetic acid as solvents was respectively located approximately between R_f 0.7 and 0.8, 0.5 and 0.9, and 0.7 and 0.8. Each value is the average value of the front or back line of the zone, since none of the boundaries was uniform. However, all the bitter zones had the same appearance and correspond to the spot closest to the front on the paper strip previously described. They were yellow under VL and bright bluish-yellow under UV-L.

The bitter fraction extracted from the bitter zone was more genuinely bitter than before chromatography. It was evident that most of the taste-interfering compounds as well as many fluorescent impurities had been removed from the crude bitter principle by paper chromatography.

In the preliminary investigation, yield of the crude bitter principle from the bitter zone of each chromatogram was so small that it was only sufficient for positive taste testing. For isolation of the crude bitter principle in larger quantity by paper chromatography, 300-g batches of celery powder were each extracted with 1.5 L technical-grade chloroform in a 4-L separatory funnel. After the solvent had been partially removed under vacuum from the extract, purified sand was added and the solvent was then removed completely. The residue coated on sand furnished a large surface for water extraction. The water extract was evaporated to dryness at 40°C. The residue left after evaporation was again extracted with water. Much of the tasteless impurity coagulated and remained undissolved. The water solution was filtered through Celite to remove most of the gelatinous material, and then extracted with petroleum ether to remove the remaining trace of chlorophylls. The bitter water filtrate was concentrated and chromatographed as described, but on larger sheets of filter paper. The infrared absorption spectrum of the bitter fraction from the bitter zone of the chromatogram developed with one solvent system was compared with those from the bitter zones developed with other solvents. They were not similar.

Further studies were conducted by chromatographing concentrated bitter extract of celery with water, BAW, and finally 2% acetic acid. The yellow amorphous material recovered from the bitter zone of the last chromatographic system was intensely bitter. However, the infrared absorption spectrum of the material thus obtained from one batch of celery still differed from that prepared from the other, though the spectra had shown greater similarities. Various paper chromatography systems were tested, but no constancy of the infrared absorption spectrum could be achieved. It was evident that this bitter fraction could not be further resolved by the methods of paper chromatography under investigation.

This bitter fraction was then submitted to electrophoretic analysis. Preliminary studies were conducted on paper strips with buffer solutions of different pH values. It was found that, with phosphate buffer solution of pH 7.6, the bitter fraction was resolved into three bands. Two bands, one bright grayish-blue fluorescent under UV-L and the other yellow under VL, moved toward the anode; the third band, faintly purple under UV-L, moved toward the cathode. With the curtain continuous-flow electrophoresis apparatus, the bitter fraction was resolved in the same buffer solution into three components in quantities large enough for organoleptic sampling. The purple band cor-

responded with the cationic band on the paper strip. The collected fractions were tasted. Those from the purple band were intensely bitter, and those from the other bands were not bitter.

In the later study, 1-kg batches of chopped fresh celery were each placed in 1 L of boiling 95% ethanol and refluxed. To remove alcohol completely, the extract was evaporated to dryness in a rotatory flash evaporator. The residue was dispersed in water and extracted repeatedly with petroleum ether to remove chlorophylls and some other materials. The emulsion formed was broken by centrifuge. The water solutions thus prepared from 10 batches of celery were joined, concentrated to 100 ml under vacuum, and filtered through Celite. The clear solution was submitted to a rapid crude separation by curtain electrophoresis using ammonium carbonate as electrolyte (Consden *et al.*, 1947). Crude separation was repeated on the bitter material contained in the bitter fraction collected. The bitter material in the bitter fraction recovered from the second crude separation was dissolved in the same electrolyte solution for slow, fine separation (Fig. 1-a). The purple fluorescent fraction collected from the second fine separation (Fig. 1-b) was lyophilized. After removal of water, ammonium carbonate was completely distilled off by heating the residue under vacuum at 50°C. After a little water was added and then evaporated, the amorphous residue crystallized into fine white needles, colorless under the microscope (Fig. 2).

The crystalline compound melts sharply at 159°C, and has a "bitterness threshold" of 90 in comparison with 150 of naringin (given as 100 ppm in Merck Index, 7th ed., p. 708 (1960), Merck & Co., N. J.; sample, courtesy of Mr. J. W. Kesterson), and 30 of pure quinine (quinine, N.F., manufactured by New York Quinine & Chemical Works, Inc., N. Y.). Bitterness threshold is defined herein as the minimum concentration (ppm) at which bitterness of a bitter compound is barely noticeable. The compound isolated is the bitter principle of celery. The average yield from 10 kg of fresh celery petioles was 17 mg.

It is soluble in water, methanol, and ethanol; slightly soluble in technical-grade chloroform, which contains ethanol as stabilizer; and insoluble in pure dry chloroform, ethyl acetate, anhydrous diethyl ether, *n*-hexane, carbon tetrachloride, and carbon disulfide.

The bitter principle is negative to ferric chloride and magnesium-hydrochloric acid tests (Geissman, 1955). The pyrolysis test is positive to acetaldehyde (Feigl, 1956).

The infrared absorption spectrum (Fig. 3) has major maxima at wave numbers 3340, 2940, 1840, 1420, 1080, 1020, 930, and 885K. The maxima 3340 and 1840K indicate –OH group(s) (Gilman, 1953), and the maximum 2940K indicates C–H group(s) (Gilman, 1953). The spectrum does not indicate an unusual functional group.

There is no absorption maximum between wavelengths 340 and 750 mμ. The ultraviolet absorption between wavelengths 200 and 340 mμ is given in Fig. 4. There are two maxima at 201 and 237 mμ, and an inflexion at 300 mμ.

The compound could be hydrolyzed with 2*N* hydrochloric acid (Power and Salway, 1913). D-Glucose was identified by paper chromatography in three solvent systems, BAW, *n*-butanol-pyridine-benzene-water (5:3:1:3, Petrelli *et al.*, 1960), and ethyl acetate-pyridine-water (3.6:1:1.15; Colombo *et al.*, 1960). The bitter principle is therefore a D-glucoside.

DISCUSSION

During isolation of the bitter principle, every fraction separated out was tasted to be sure that no detectable bitter taste was escaping notice. When an organic solvent was used, it was removed by rotary flash evaporation, and the residue was tasted either directly or after being mixed with water. None of the volatile compounds of the celery was bitter, and no bitterness could be steam-distilled from the celery. Molecular distillation was also employed, but the distillate was not bitter. Bitterness, however, was extracted completely into chloroform of technical grade, which contains ethanol as stabilizer, because of the excessive amount of the solvent used. The extracted celery powder was further extracted successively with ethanol and with water. No bitter taste was detectable in compounds extracted by these solvents. The pulp was tasted, it was not bitter. These facts indicated that the bitter principle isolated is the only detectable bitter principle of the celery under investigation.

Although organoleptic sampling cannot be a rigorous test for the purity of a chemical compound, it was nevertheless essential as a criterion during the present preliminary process of isolation since, if the compound isolated is not bitter it is not a bitter principle, no matter how pure it may be chemically.

The minuteness in quantity of the bitter principle in celery is understandable, because bitterness in celery was associated mainly with the dark-green outer layer of the petiole (Hall, 1959). After laborious removal of

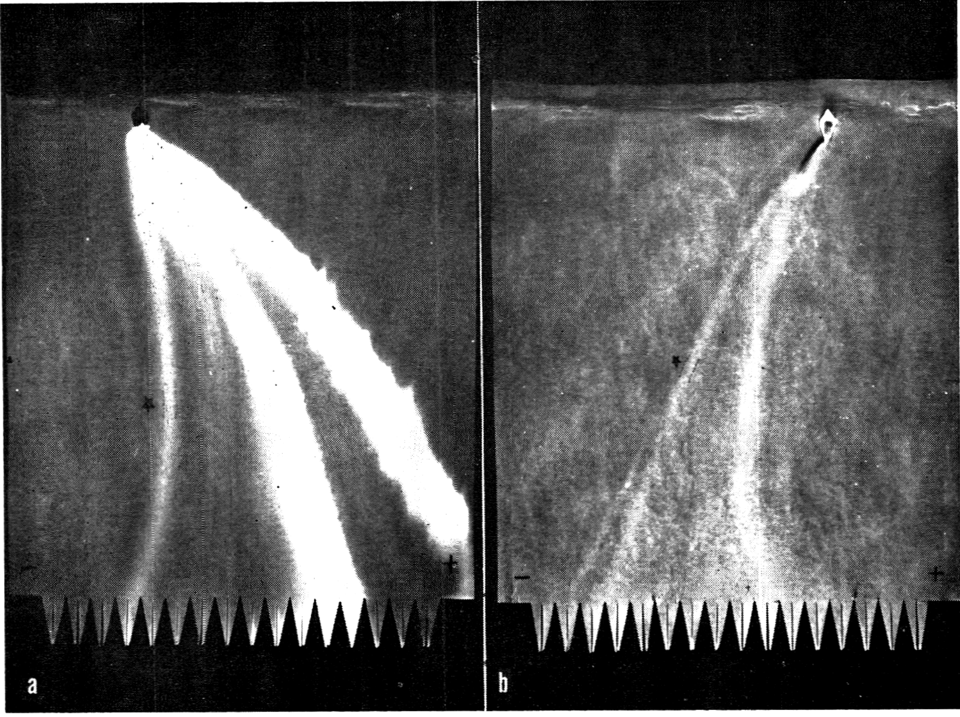

Fig. 1. Isolation of the bitter principle of celery by curtain electrophoresis. a) Electrophoresis of bitter material obtained from crude separation of bitter concentrate. Concentration of ammonium carbonate, 0.48 g per L, pH 7.88. Current, 27 ma. Setting: sample feeding rate 4 (1.7 cm ID tube), cathode wick 6, anode wick 5, overflow 8. *Bitter band, grayish-blue under UV-L (ultraviolet light of long wavelengths). b) Electrophoresis of the bitter fraction from (a). Same electrolyte solution. Settings the same except cathode wick 5, anode wick 6. *Bitter band containing pure bitter principle, faintly purple under UV-L. Narrow independent streak left to bitter band, remainder of bitter band formed at a higher voltage.

this thin layer, the petiole of the bitter celery tasted bitter only when the celery was intensely bitter. Since the dark-green outer layer is only a very small fraction of the petiole, the quantity of the bitter principle in celery cannot be more than minute.

The bitterness of celery despite its containing so little bitter principle of but limited potency can be explained by the immediate contact of the bitter principle with taste buds. When we chew a petiole of bitter celery, we bite into the outer layer first. The bitter principle released by such an impact evidently contacts the taste buds immediately, imparting the bitter sensation without first dispersing itself in the large bulk of the unbitter part of the petiole. In other words, only a minute quantity of the principle is sufficient to give the bitter sensation. Finally, although the bitter principle is much more soluble in methanol than in technical-grade chloroform, methanol was not a practical solvent for extraction of the bitter principle in the preliminary study. The bitterness was masked in the methanol extract by compounds that may be more soluble in methanol than the bitter principle. This fact is further evidence not only of the minute quantity of the bitter principle but also of the necessity of immediate contact of the bitter principle with taste buds in order to impart the bitter sensation.

The finding that the quantity of the bitter principle in bitter celery was so minute required exhaustive study. That only a minute quantity of a bitter principle of limited potency can be so bitter is a new conception. Localization of the bitter principle where it makes immediate contact with taste buds is probably the explanation.

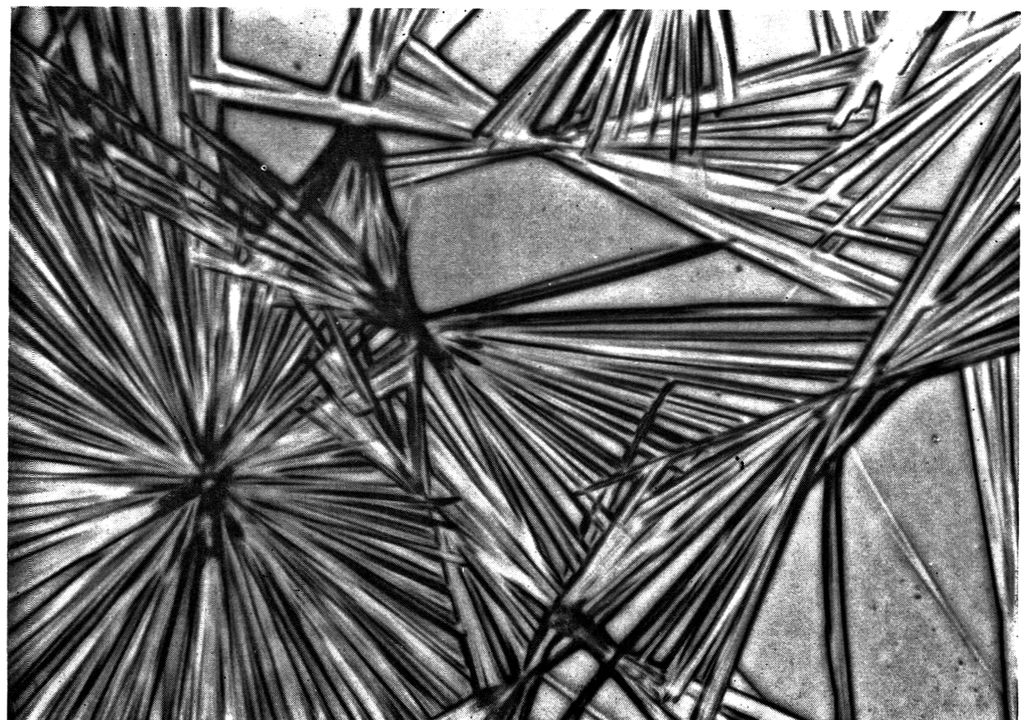

Fig. 2. Agglomeration of the crystals of the bitter principle of celery. Colorless crystal formation after rapid evaporation of water. 560×.

The yield in the experiment should not be taken as the actual content of the bitter principle in the celery. Loss must have been considerable and inevitable during the complicated isolation because, as a rule, loss is considerable when a compound is isolated with even an established procedure consisting of a number of processes.

The positive test of acetaldehyde in pyrolysis agrees with the fact that the compound is a glycoside. The negative tests with ferric chloride and magnesium–hydrochloric acid indicate that the compound is not one of the known glycosides of celery and the closely related parsley (Hudson, 1949). Those glycosides, flavonoid compounds, are positive to the two tests. The bitter principle is therefore a so far unknown glycoside of celery.

Electrophoresis for isolation of the bitter principle may have opened up a new way of isolating other elusive bitter principles in fruits and vegetables.

The structure of the bitter principle is being studied.

ACKNOWLEDGMENT

I am grateful to Dr. R. A. Dennison and Dr. C. B. Hall for suggesting this problem. I am also indebted to Mr. Billy Rogers for a generous supply of celery; to Mr. G. J. Edwards for spectral analyses; to Dr. H. C. Harris and Dr. S. H. West for the use of electrophoresis apparatus. Finally, I wish to acknowledge the technical assistance of Mr. S. R. Christie and Mr. J. J. Tierney.

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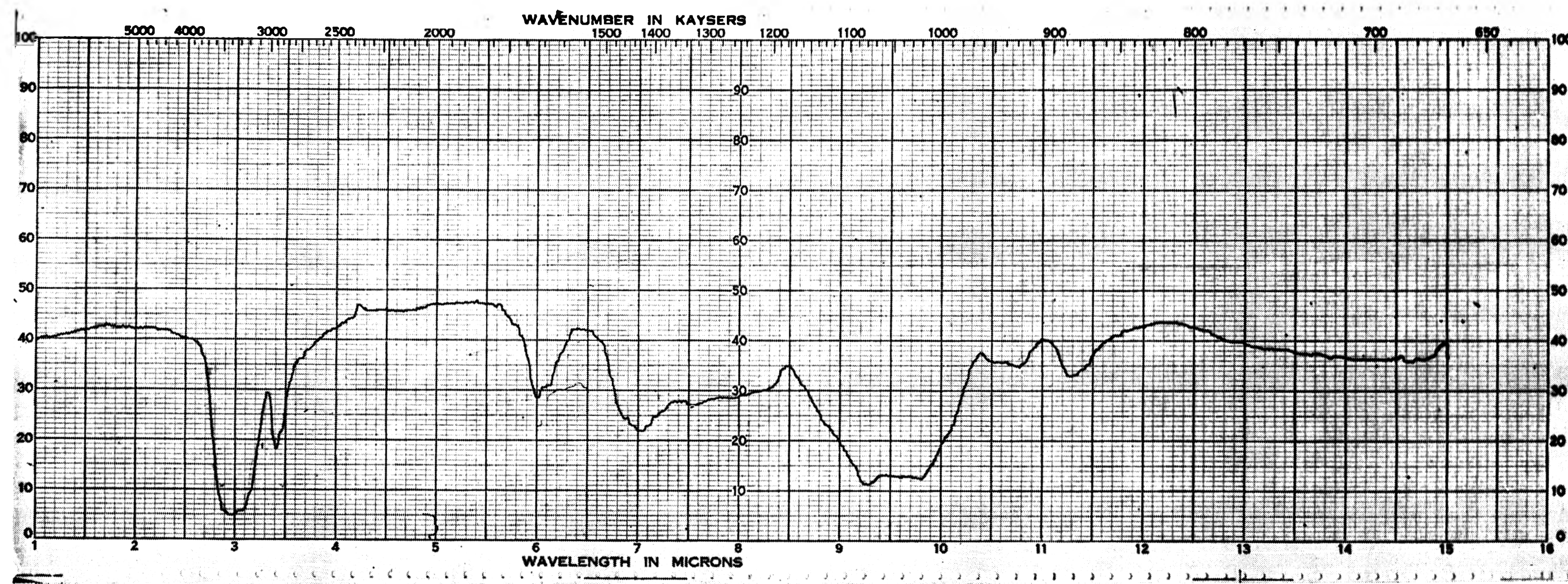

Fig. 3. Infrared light absorption spectrum of the bitter principle of celery. Prepared with double-beam Beckman–IR4; as film on sodium chloride plate.

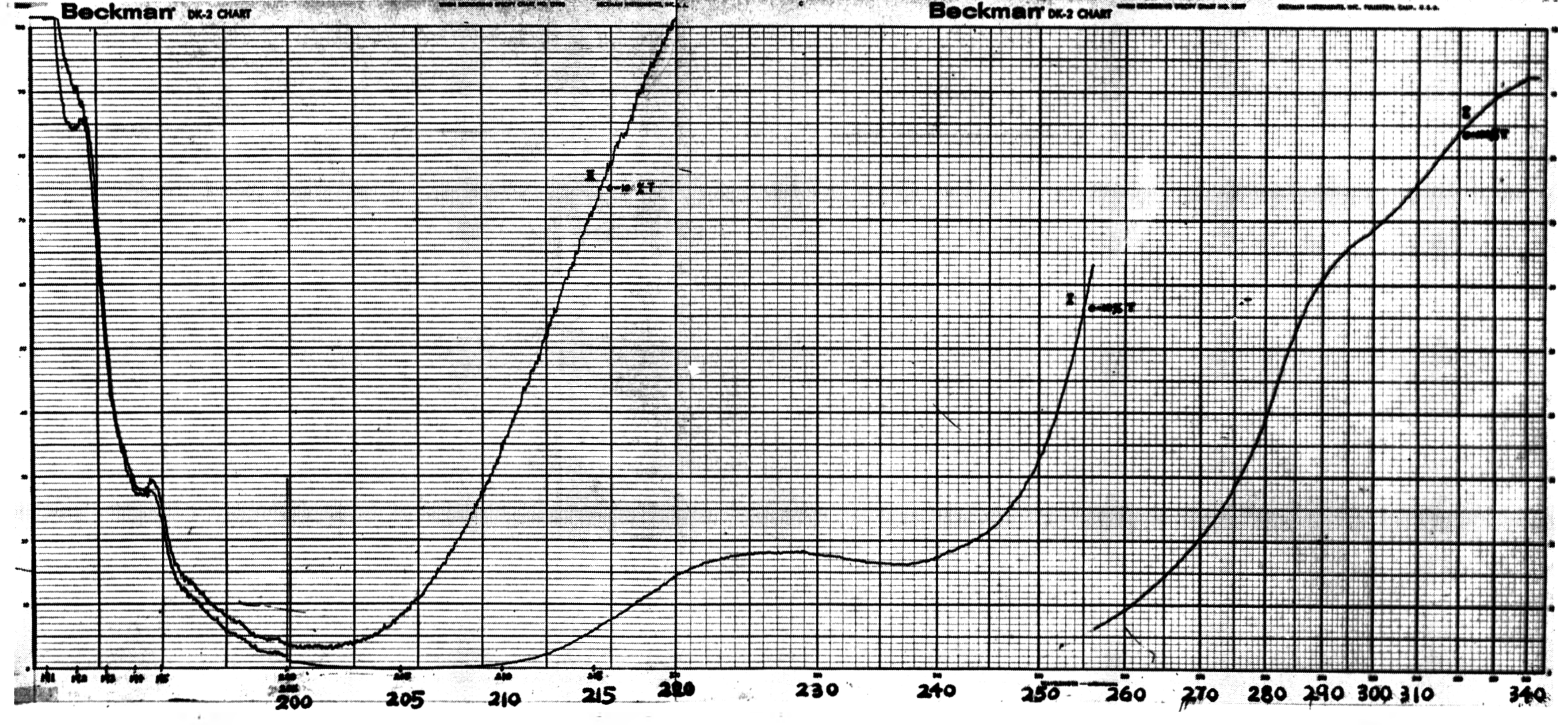

Fig. 4. Ultraviolet light absorption spectrum of the bitter principle of celery. Prepared with Beckman Ratio Recording Spectrophotometer DK-2. Concentration in water: I, 1.92 mg/ml; II, 1.10 mg/ml.

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Radioresistance of Five Strains of *Clostridium Botulinum* in Selected Food Products[a, b]

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(Manuscript received October 5, 1960)

SUMMARY

Spore recovery counts made on five strains of *Clostridium botulinum* indicated striking differences in radioresistance. Strain 12885A was most resistant, and strain 32B least resistant. A difference was noted in the survival of spores in five food products (green beans, chicken, codfish, pork, and beef), with green beans providing the most destruction. It was also observed that a given substrate was found to allow a greater per cent survival of one strain than another, so that two strains compared in one food do not always show the same relationship as when compared in another food. Some evidence indicates a modest recovery of viability of the irradiated spores during frozen storage prior to culturing. Since the character of the food determines in part the dose required for the destruction of *Cl. botulinum*, no single dose would be best for all food products.

The first large-scale study of the effect of irradiation on *Clostridium botulinum* in various food products was begun in 1955. The program, undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, was of such scope that the research laboratories of the National Canners Association, American Can Company, and Continental Can Company all collaborated in the work. The first two laboratories have published reports on their phases of the investigation, one by Denny *et al.* (1958) and the other by Pratt *et al.* (1959). Those papers described the over-all program and the results of work carried out using neutral phosphate and certain food products as the substrates during irradiation. The foods reported on were peas, chicken soup, and pork, and the inoculum was a mixture of 10 strains of *Cl. botulinum*.

As an outgrowth of this work, additional programs were instigated by the Quartermaster Food and Container Institute to find missing links of fundamental information on the reaction of *Cl. botulinum* to destruction by irradiation. This paper presents an investigation designed to define further the influence of the food substrate on the radioresistance of the spores of *Cl. botulinum*. Selected for study were 5 foods of particular interest to the armed forces. These were green beans, chicken, codfish, pork, and beef. The resistance to irradiation of 5 selected strains of *Cl. botulinum* inoculated into these foods was determined by the procedure described below.

Determining the irradiation dose for these additional foods was expected to clarify whether a single dose procedure would be practical for foods generally; or whether it would be necessary to plan for specific radiation treatments for different foods. From a more theoretical standpoint, the experiment was also designed to determine whether the food substrate altered the radioresistance of different strains of *Cl. botulinum* to the same degree.

EXPERIMENTAL PROCEDURE

Screening the bacterial strains. In the first study (Denny *et al.*, 1958; Pratt *et al.*, 1959), the inoculum of *Cl. botulinum* spore suspensions con-

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[b] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, QM Research and Engineering Command, U. S. Army, and has been assigned No. 2036 in the series of papers approved for publication.

sisted of mixed spores from 5 strains of Type A and from 5 strains of Type B. To gauge their radioresistance, the different strains were subjected to a screening test. Spore suspensions produced by growing in beef heart infusion–casein medium (pH 7.1) for 12 days at 98°F (37°C) were collected by centrifuging. After resuspending in a small amount of the natural liquor, the suspensions were heated 10 minutes at 170°F (77°C), cooled, and refrigerated.

For the screening tests, the spore suspensions were standardized to yield about 1×10^6 spores per ml when added to pork-pea infusion (pH 7.4). Five-ml portions of these standardized suspensions were filled into polystyrene vials, frozen, and irradiated frozen at .5, 1.0, 1.54, and 1.85 Mrad. A mixture of fission products in spent fuel elements was used for the gamma source. Subsequently, counts were made using pork-pea agar to ascertain the number of surviving spores. The counts obtained are shown in Table 1. Based on these results, strains 78A, 13824A, 12885A (non-toxic) and strains 12033B and 32B were selected for use in the experiment. The non-toxic strain, 12885A, was incorporated in the program since the results indicated it possessed the greatest radioresistance among those tested.

Inoculum. The spores prepared for the screening tests and suspended in the natural liquor were used for the experimental inoculum. The known spore concentration of any given stock suspension was used in calculating the volume of suspension to be added to a given volume of product, to result in a final concentration of 1×10^7 spores per gram of product. This volume of inoculum was then diluted with sterile water amounting to 10% of the weight of the food to facilitate mixing and insure adequate blending with the prepared food.

Preparation of foods. *Green beans.* Blanched frozen beans were thawed and passed through a Fitzpatrick comminuter. No water was added, since the consistency was acceptable.

Chickens. Frozen boned chicken was thawed and ground in a meat grinder through a ⅜-in. plate. It was then placed in pans in a retort and heated under steam pressure until a minimum of 174°F (79°C) was reached, in order to inactivate enzymes.

Codfish. After being thawed, the frozen product was placed in a wire basket in a steam retort and heated under pressure to a minimum of 155°F (69°C), during which time the pieces disintegrated into flakes.

Pork. This frozen ground product was handled by heating in pans in a steam retort to 200°F (94°C).

Beef. The ground beef, after thawing, was heated in steam to a minimum of 166°F (75°C).

After heating, all foods were cooled to −20°F (−29°C) and held there until used.

Packing of cans. Before the time scheduled for packing the respective foods, each was held 8 hours at 40°F (4°C) for thawing. The required amount of thawed product needed to fill the number of cans in a given lot was then weighed into a laboratory-model vacuum meat mixer. The inoculum, as described above, was thoroughly blended with the food by mixing for 12 min under 26 in. of vacuum. For control cans, the same volume of sterile water (10%) was vacuum-mixed with product as was added to all inoculated lots. Baby-food-size cans (2⅛ in. diam by 2⅛ in. tall; 202 × 202) were filled with 90 g of food from the mixer, the top sealed on by double-seaming, and the contents frozen at −20°F (−29°C). All control lots of uninoculated products were packed be-

Table 1. Irradiation screening tests: Per cent survival of individual strains of *Clostridium botulinum* at 5 doses (Mrad).

Strain	0	0.5		1.0		1.5		1.8	
(Type A)	Count/ml	Count/ml	%	Count/ml	%	Count/ml	%	Count/ml	%
62	820,000	96,000	11.7	3,400	.41	78	.0095	3.6	.00043
73	1,420,000	224,000	15.8	3,200	.22	84	.0059	6.0	.00042
78	1,040,000	194,000	18.6	4,000	.38	68	.0065	2.4	.00023
12885	1,000,000	176,000	17.6	16,800	1.6	1,020[a]	.102	53.2	.0053
13824	820,000	162,000	19.8	4,800	.58	[b]		11.6	.0014
(Type B)									
12033	860,000	153,000	17.8	73	.0085	5.4	.00063	.8	.00009
13983	1,560,000	300,000	19.2	236	.015	6.6	.00042	.4	.000025
32	1,930,000	49,600	2.5	42	.0026	3.4	.00017	.4	.00002
213	1,100,000	126,000	11	33	.003	2.6	.00023	1.2	.0001
113	1,100,000	136,000	12	186	.016	32.6	.0029	.4	.00003

[a] Single-colony isolates checked for toxicity: non-toxic.
[b] Count not recorded, since vial had filled with liquid and, when frozen, the cap was forced off.

Table 2. Number of cans packed for irradiation program.

	Storage and examination	Products					
		Beans	Chicken	Fish	Pork	Beef	Total
Uninoculated cans							
0 Mrads	Held frozen for subculture	6	6	6	6	6	30
0.85 Mrads	Incubated at 86°F	6	6	6	6	6	30
1.7 Mrads	Incubated at 86°F	6	6	6	6	6	30
Total uninoculated		18	18	18	18	18	90
Inoculated cans [a]							
0 Mrads	Held frozen for subculture	30	30	30	30	30	150
0.85 Mrads	Held frozen for subculture	30	30	30	30	30	150
1.7 Mrads	Held frozen for subculture	30	30	30	30	30	150
Total inoculated [a]		90	90	90	90	90	450

[a] Five different strains of *Clostridium botulinum* spores are represented. Eighteen cans of each food product were inoculated with each strain, six of these for each radiation dose.

fore preparation of the inoculated variables. Table 2 shows the number of cans packed of each product for each inoculation and irradiation variable.

Irradiation. The irradiation was carried out at the Dugway Proving Grounds of the Army, Dugway, Utah. Ceric-cerous dosimetry was used to determine the dose rate. All cans were held frozen before and during irradiation. Freezing conditions were maintained in the exposure unit by circulating cold ethyl alcohol through cooling coils inside the radiation chamber. Nine of the experimental cans were placed in a No. 10 can ($6^{3}/_{16}$ in. diam by 7 in. tall) fitted with a holder to maintain the position of the test cans and to prevent their rotation independently of the No. 10 can.

The equipment used to expose the No. 10 cans loaded with test cans to irradiation is described in detail elsewhere (Christensen *et al.*, 1957a, b), and a schematic drawing is shown in Fig. 1. Essentially, it consisted of a horizontal rotating tube about 7 in. in diam and 70 in. long, surrounded by spent fuel elements from an atomic reactor. The No. 10 carrier cans were pushed through the tube by a cam-actuated plunger that traveled the 7-in. length of one can. After the tube was loaded with 10 cans, each new can introduced into the tube by the plunger resulted in the discharge of a fully irradiated can from the exit end. Total dose was controlled by adjusting the speed of rotation of the plunger cam.

The irradiation plan was worked out to distribute all test-lot variables among the No. 10 carrier cans and to feed the carrier cans in such order that any unknown variables in exposure would affect each test lot similarly.

The irradiation doses selected were expected to permit some survival of spores in all test lots. This would then determine destruction rate in each given food, and indicate the effect of food substrate on the relative resistance of the test strains of *Cl. botulinum.*

Storage. All irradiated inoculated cans and the

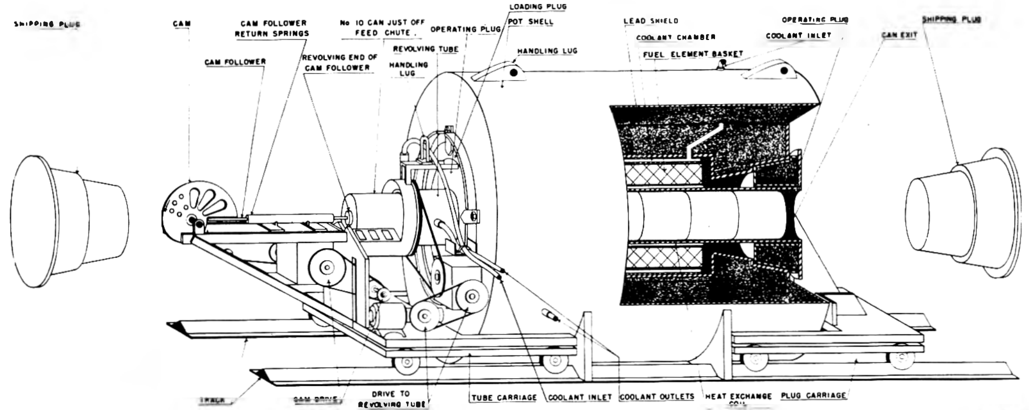

Fig. 1.

uninoculated unirradiated lots were maintained in a frozen state pending subculture for viable spore counts. The uninoculated irradiated lots were incubated at 86°F (30°C) and examined for swells over a period of one year.

Survivor counts. Viable spore counts were made on all lots of inoculated cans and on the uninoculated lots that were not irradiated. The order and sequence of sample selection were based on a 5 × 5 Graeco-Latin square design. The first series of 30 counts was made on one can of each product, inoculated with each strain, plus one uninoculated can of each product from the unirradiated lot. Then, one can of each lot receiving .85 Mrad was examined, followed by one can per lot of the high-dose (1.7 Mrad) series. With the completion of counts on one can per lot, the second can was examined following the same order. After the examination of 4 cans per variable, statistical analyses revealed the results of replicate cans to be so consistent that further count data were considered unnecessary.

The cans to be opened for viable spore counts were allowed to stand 18 hours at 40°F (4°C) and then washed with an iodine germicide. The cans were then opened aseptically, a 50-g sample removed to a sterile blender, and 200 ml sterile cold water added. Blending was carried out for 2–3 min under vacuum. From this initial dilution, additional serial dilutions were made as required, and portions of 1 ml each distributed in 5 Veillon tubes, each containing 0.4 ml of 5% sodium bicarbonate. Pork-pea agar was then added, followed by stratification with a sterile Vaseline-paraffin mixture and by incubation at 86°F (30°C). Ninety-six hours was the standard period of incubation for all residual spore count samples. For both the uninoculated and the inoculated unirradiated samples, the initial 1:5 dilution was heated 10 min at 155°F (69°C) before culturing. Table 3A presents

Table 3. Product anaerobic spore content and spoilage in irradiated uninoculated cans.

	Product natural anaerobic sport content per g [a]					Spoilage of irradiated uninoculated cans [b]	
	Can no.					Dose	
	1	2	3	4		.85 mrads	1.7 mrads
Beans	240	80	140	40	Beans	3 OK, 3 swells	6 OK
Chicken	140	60	30	4	Chicken	6 swells	6 OK
Codfish	20	16	12	16	Codfish	6 swells	6 OK
Pork	6	140	6	18	Pork	6 swells	5 OK, 1 swell
Beef	140	200	70	40	Beef	6 swells	2 OK, 4 swells

[a] Samples heated 10 min at 155°F (68°C).
[b] Based on incubation at 86°F (30°C).

Table 4. Average counts (4 cans) per gram and per cent survival, 5 strains of *Clostridium botulinum* on 5 foods.

	78A		13824A		12885A		12033B		32B	
Food [a]/dose [b]	Count	% surv.	Count	% surv.	Count	% surv.	Count	% surv.	Count	% surv.
B/0	4,170,000		10,450,000		8,800,000		14,150,000		12,400,000	
B/L	1,050,000	25	1,965,000	18	2,160,000	24	445,000	3.1	155,500	1.2
B/H	490	.011	189	.0018	8,450	0.96	239	.0016	9.5	.000075
C/0	1,750,000		6,150,000		4,950,000		3,400,000		3,060,000	
C/L	377,500	21	1,865,000	30	1,690,000	34	217,500	6.3	97,000	3.1
C/H	662.5	.037	1,745	.028	15,550	.31	750	.022	36.5	.0011
F/0	3,500,000		10,850,000		5,800,000		9,950,000		6,250,000	
F/L	775,000	22	677,500	6.1	895,000	15	75,500	.75	22,250	.35
F/H	3,115	.088	520	.0047	8,500	.14	365	.0036	125	.002
P/0	1,950,000		8,550,000		4,350,000		4,550,000		3,150,000	
P/L	750,000	38	2,365,000	27	1,825,000	42	248,000	5.1	72,500	2.3
P/H	1,380	.07	1,555	.018	17,600	.404	565	.012	69.5	.0022
S/0	2,535,000		9,450,000		5,850,000		4,780,000		2,385,000	
S/L	1,230,000	48	2,025 000	21	2,065,000	35	296,500	6.2	92,500	3.8
S/H	3,130	12	2,750	.029	18,700	.31	810	.016	92.5	.0030

[a] B, beans; C, chicken; F, codfish; P, pork; S, Beef.
[b] O, no dose; L, .85 Mrads; H, 1.7 Mrads.

the natural spore counts for the food products packed (uninoculated unirradiated samples); Table 3B presents the spoilage data for the irradiated uninoculated lots.

Toxicity tests. Toxicity tests were made on a limited basis, using only colonies obtained from the highest dose level from each product and each of the 5 strains exposed. The isolated colonies were grown in Reed-Orr medium. After a minimum incubation time of 1 week at 98°F (37.5°C), 0.5 ml of the culture was injected intraperitoneally into a mouse. Toxic cultures were found in all instances except from the strain 12885A.

RESULTS AND DISCUSSION

Table 4 shows the average residual *Cl. botulinum* spore counts for all cans examined, and the per cent survival. The fraction of organisms surviving .85 Mrad was generally rather high and provided little contrast between survival rates of the various strains in the various products. The fraction of organisms surviving 1.7 Mrad was much more sensitive to the effect of product substrate, and the discussion is directed to the data from this dose level.

The fraction surviving is the count per gram in the irradiated product (average of 5 tubes) divided by the count per gram in the same product not irradiated (average of 5 tubes). All such data were "normalized" by logarithmic transformation and submitted to three-factor analysis of variance (Snedecor, 1956). The results are in Table 5. The

Table 5. Analysis of variance: logarithms of fraction of spore count surviving 1.7 Mrad.

Source of variation	Degrees of freedom	Mean square	Test	
Organism	4	14.775	**	
Product	4	4.097	**	
Time	3			
Linear	1	.4147	*	
Quadratic	1	.0027	n.s.	
Cubic	1	.0125	n.s.	
Organism × product	16	.309	**	
Organism × time	12	.082	n.s.	pooled error: .0600
Product × time	12	.0669	n.s.	
Organism × product × time	48	.0526		
Total	99			

* Significant at 5% level.
** Significant at 1% level.

following conclusions were drawn from the analysis:

1) There were obvious differences in radioresistance among the five strains, significant at the 1% level. As shown in the table below, 12885A was the most radioresistant of the strains tested, and 32B the least radioresistant.

Strain	Av [a] % surviving
12885A	$23. \times 10^{-2}$
78A	$5. \times 10^{-2}$
13824A	0.98×10^{-2}
12033B	0.81×10^{-2}
32B	0.12×10^{-2}

[a] Geometric mean for all five food products shown in Table 4.

2) The degree of destruction caused by irradiation varied with the substrate (significant at 1% level), being greatest in green beans.

Substrate	Av [a] % surviving
Beef	4.4×10^{-2}
Pork	2.9×10^{-2}
Chicken	2.4×10^{-2}
Fish	1.3×10^{-2}
Green Beans	0.30×10^{-2}

It might be assumed that green beans offer less protection against radiation because of their lower pH value. This would be in accordance with experience in thermal processing as reported by Sognefest *et al.* (1948). Reports indicate, however, that pH does not affect the radioresistance of bacterial spores (Pratt and Ecklund, 1954; Proctor *et al.*, 1955).

3) A given substrate may permit greater destruction of one strain than another, so that two strains compared may not show the same relationship in one food as in another (significant at 1% level). The average per cent of each strain surviving in each product is tabulated below. To determine whether there is a real difference between two averages in this table, divide the larger average by the smaller of the two. If this ratio exceeds 2.9, the two averages are significantly different at the 1% level.

Examination of the table will show a number of instances where the order of re-

sistance in the various food substrates differs between the strains studied.

Substrate	Av.[b] % surviving (multiplied by 100) 12885A	78A	13824A	12033B	32B
Beef	32	14	3.0	1.9	0.60
Pork	41	7.2	1.7	1.4	0.28
Chicken	39	3.0	2.5	2.4	0.12
Fish	15	8.3	0.42	0.36	0.20
Green Beans	9.6	1.2	0.18	0.15	0.0076

[b] Geometric mean.

4) The change in fraction of surviving organisms with time in frozen storage was significant at the 5% level, as shown in the table below. This may be evidence for recovery of some irradiated spores during frozen storage.

Order of culturing	Av.[b] % surviving (25 cans)
1st set cultured	1.3×10^{-2}
2nd set cultured	1.6×10^{-2}
3rd set cultured	1.7×10^{-2}
4th set cultured	2.0×10^{-2}

5) As noted in Table 3, swell spoilage developed in uninoculated cans of beef and pork given the highest irradiation dose (1.7 Mrad). The beef, showing 67% spoilage, had a higher natural load of anaerobic spores than did the pork, which showed 17% spoilage. However, 200 was the highest count found in these two foods.

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Chemical Composition of Potatoes. I. Preliminary Studies on the Relationships Between Specific Gravity and the Nitrogenous Constituents

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SUMMARY

Katahdin potatoes (1959 crop) were placed in storage at 38°F. Monthly samples were removed and separated into three specific-gravity levels—high, intermediate, and low. Total solids changed but little in storage, indicating that shrinkage is due to a loss of both solids and water in the ratio in the original composition. Total and soluble nitrogen analyses over 10 months demonstrated an inverse relationship between total solids and these constituents when calculated on a moisture-free basis. The nitrogen per gram of fresh weight shows no significant difference between samples of different solids contents. Therefore, the apparent inverse relationship on a moisture-free basis is due to the storage of other constituents, presumed to be principally starch, in the case of high-solids potatoes. About 60–62% of the nitrogen is extracted by 70% by weight ethanol. Subsampling of large lots of potatoes for specific-gravity studies is extremely difficult. All data should be checked for variations from sampling error.

Studies on the nitrogenous constituents of potatoes have been reported by several researchers. As noted (Neuberger and Sanger, 1942), their results are difficult to interpret since the nitrogen value recorded in most of the experiments was total nitrogen only. Neuberger and Sanger (1942) attempted to characterize the different nitrogenous fractions and reported data for a number of varieties showing fairly wide variations in total nitrogen and in the relative proportions of the different fractions.

Other workers, including Lampitt and Goldenberg (1940), have reported total nitrogen analyses obtained on potatoes from different countries. Representative of studies on the nitrogenous fractions are papers by Steward and Street (1946) and Kaspers (1959) on amides; Dent *et al.* (1947), Steward *et al.* (1949), Zacharius *et al.* (1952), Kaspers (1959) and Szalai (1959) on amino acids; and Osborne and Campbell (1896), Groot (1946), and Chick and Slack (1949) on the proteins.

Problems of extraction of potatoes for amino acid analyses were discussed by Talley *et al.* (1958), who showed that, unless continued and complete extractions were made with 70% by weight ethanol, the picture of the relative amounts of the amino acids would be distorted by differences in rate of extraction of the several amino acids.

None of these reports attempted to show the relationships of specific gravity (total solids) and the composition of potatoes. Since a greater percentage of each crop is processed, this relationship is important because of the use of specific gravity as a measure of suitability for a specific product. This paper demonstrates the relationship of specific gravity and the nitrogenous constituents of potatoes, and points out some of the problems involved in such studies.

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MATERIALS AND METHODS

Katahdin potatoes, grown during the 1959 season at the University of Maine Agricultural Experiment Station, Presque Isle, Maine, were furnished by the University. One ton of potatoes, the controls for an experiment on methods of killing tops, were stored at 38°F in 60-lb boxes selected on a random basis in one of the University storage houses beginning September 25, 1959. A sample consisting of two of these boxes was immediately sent to Philadelphia to determine the initial composition. Each month for nine consecutive months, a 120-lb sample was removed from storage and sent to our laboratory for analysis. Table 1 presents the data of sampling, average specific gravities, moisture values and relative weights of the samples.

Table 1. Weight-volume data for 38°F storage samples.

Date of sampling	Time (months)	Av specific gravity (potato hydrometer)[a]	Moisture [b]	Relative weight (measure of shrinkage)
Sep. 25, 1959	0	1.066	83.1	100.0
Oct. 16	1	1.070	82.2	98.5
Nov. 17	2	1.065	83.3	98.4
Dec. 28	3[c]	1.066	83.1	98.1
Jan. 18, 1960	4	1.069	82.4	97.3
Feb. 17	5	1.067	82.8	97.3
Mar. 17	6	1.066	83.1	95.4
Apr. 18	7	1.065	83.3	94.6
May 14	8	1.068	82.6	94.5
June 16	9[d]	1.065	83.3	93.1

[a] Taken on 8-lb sample from shipment before separation into the several specific-gravity fractions.

[b] Read from hydrometer conversion graph.

[c] First December shipment (December 13), frozen in transit.

[d] This sample removed from 38°F storage on May 16, 1960, and placed in 45°F storage until June sample taken. Definite evidence of sprouting.

Upon arrival, the average specific gravity of an 8-lb sample was determined with a N.P.C.I. (National Potato Chip Institute) potato hydrometer. Using this specific gravity as the median, the samples were separated into six fractions with salt brines of the appropriate concentration. Each fraction represented a range of 0.003 specific-gravity units, as shown in Table 2. The highest, an intermediate, numbered 3, and the lowest specific-gravity fractions were selected for analysis. The three other fractions were discarded. Samples for analysis were taken by arranging each fraction according to size of potatoes and selecting an equal number of tubers of each size.

Table 2. Example of ranges of specific gravity.[a]

Fraction	Specific gravity range [b]	Selected for analysis	% of sample	Av sp gr (hydrometer)
1	>1.072	yes	11.00	1.078
2	1.069–1.072	no	13.07	1.072
3	1.066–1.069	yes	24.59	1.069
4	1.063–1.066	no	16.72	1.067
5	1.060–1.063	no	14.40	1.063
6	<1.060	yes	20.22	1.060

[a] Sample No. 1, September.

[b] These ranges are the limits of the specific gravities of the salt brines employed.

The samples from each fraction were peeled for 20 seconds in an abrasive peeler (Toledo Vegetable Peeler, Model No. A1-15). The eyes were not removed, but damaged spots were hand trimmed. Average peeling loss was about 6%. Potatoes were peeled so the samples would be nearer to the condition of potatoes when processed commercially or prepared for home use.

Each sample was extracted with 70% by weight ethanol based upon the method of Talley *et al.* (1958). An accurately weighed sample (1200–1400 g) of peeled potatoes was placed in a 1-gal Waring Blendor, and sufficient absolute ethanol was added (about 2400 g) to make 70% by weight ethanol when mixed with the water in the potatoes. With a dipper, accurately weighed aliquots of the slurry were removed after the grinding operation (1.5 min at high speed, then slowed to minimum running speed) for determination of total nitrogen and total solids and for extraction for amino acid, organic acid, and soluble nitrogen determinations. For the extraction sample, the aliquot removed corresponded to 50 g fresh weight of potatoes. This sample was first carried through 5 batchwise extractions with 70% by weight ethanol and finally through two 24-hour Soxhlet extractions. The combined extracts were concentrated in rotary evaporators at less than 40°C. The extract was then made to 500 ml with water and ethanol so that the resulting solution was 20% by weight ethanol (self-preserving).

Total solids were determined on about 18 to 20 g accurately weighed slurry samples (equivalent to 4 to 7% solids in the slurry). Drying for 2 hours at 60°C in a mechanical convection oven was carried out to remove the ethanol and part of the water; then 3 hours at 130°C resulted in constant weight.

Total nitrogen was determined by micro-Kjeldahl digestion using mercuric oxide as a catalyst and distillation by means of the A.S.T.M. official distillation equipment into saturated boric acid followed by titration with standard acid solution. To minimize foaming during digestion of the

slurries, about 5 ml of distilled water and a few drops of concentrated sulfuric acid were added to each sample, and the samples were boiled down until the residue began to brown. The catalyst and sulfuric acid were then added, and the digestion continued in the usual manner (Burroughs, 1960).

Soluble-nitrogen determinations were made by the same procedure, using aliquots of the extract prepared for amino acid and organic acid determinations.

Insoluble nitrogen (protein nitrogen) was calculated from the difference between total nitrogen and extractable nitrogen.

All analytical data are the result of at least two replicates.

The amino acid and organic acid compositions of these samples will be reported in future papers.

RESULTS AND DISCUSSION

Table 1 shows a progressive loss in weight during storage. The lack of change in solids content over the same period indicates that both solids and water are lost in the ratio of the original composition. This verifies the observation of Treadway *et al.* (1949) on the effects of storage on starch and sugars. Therefore, conclusions based on relative solids in the different specific-gravity fractions are valid.

Tables 3, 4, and 5 present the data on solids and nitrogen obtained for the high, intermediate, and low specific-gravity fractions. The June sample had been placed in 45°F storage at the time of the May sampling, which accounts for the small changes in nitrogen composition.

The 6.9% shrinkage indicated in Table 1 should result in a loss of solids of about 1.4%. This in turn should result in a gain in total nitrogen of about 0.02% during

Table 3. The solids and nitrogen composition of the high-specific-gravity fraction.

Month	% solids	% of sample	% total N (MFB)	mg total N/g fresh wt	% N in extract (MFB)	mg extractable N/g fresh wt	% insol N (MFB)	mg insol N/g fresh wt	Insol N / Total N (MFB)
Sep.	19.85	11.00	1.79	3.44	0.99	1.97	0.80	1.47	0.45
Oct.	19.20	10.30	1.77	3.38	1.03	1.96	0.74	1.42	0.42
Nov.	18.28	19.37	1.72	3.08	1.08	1.92	0.64	1.16	0.37
Dec.	18.77	22.51	1.72	3.22	1.12	2.09	0.60	1.13	0.35
Jan.	18.85	12.95	1.72	3.24	1.13	2.12	0.59	1.12	0.34
Feb.	19.05	7.66	1.72	3.27	1.04	1.99	0.68	1.28	0.40
Mar.	18.70	10.14	1.80	3.34	1.09	2.03	0.71	1.31	0.39
Apr.	18.80	14.77	1.76	3.32	1.06	2.00	0.70	1.32	0.40
May	18.98	11.54	1.79	3.40	1.11	2.11	0.68	1.29	0.38
June	18.44	25.24	1.91	3.52	1.19	2.19	0.72	1.33	0.38
Average	....	....	1.77	3.32	1.08	2.04	0.69	1.28	0.39

Table 4. The solids and nitrogen composition of the intermediate-specific-gravity fraction.

Month	% solids	% of sample	% total N (MFB)	mg total N/g fresh wt	% N in extract (MFB)	mg extractable N/g fresh wt	% insol N (MFB)	mg insol N/g fresh wt	Insol N / Total N (MFB)
Sep.	18.04	24.59	1.86	3.36	1.05	1.89	0.81	1.47	0.44
Oct.	17.68	17.85	1.95	3.44	1.14	2.01	0.81	1.43	0.42
Nov.	16.42	18.17	1.99	3.27	1.34	2.20	0.65	1.07	0.33
Dec.	16.98	20.99	2.03	3.45	1.31	2.23	0.72	1.22	0.35
Jan.	17.43	13.74	1.76	3.07	1.19	2.07	0.57	1.00	0.32
Feb.	17.62	22.14	1.88	3.32	1.18	2.06	0.70	1.26	0.37
Mar.	17.95	18.21	2.01	3.41	1.33	2.28	0.68	1.13	0.34
Apr.	17.05	12.02	2.01	3.43	1.24	2.11	0.87	1.22	0.43
May	17.43	17.78	1.97	3.47	1.16	2.03	0.81	1.44	0.41
June	16.87	16.18	2.14	3.60	1.35	2.28	0.79	1.32	0.37
Average	....	....	1.96	3.38	1.23	2.12	0.74	1.26	0.38

storage. Statistical analysis of the data, including the duplicate and triplicate values obtained, using standard deviations, indicate that this value (0.02%) would not be significant, and the data could not be interpreted as showing this small gain.

It is apparent that total nitrogen, soluble nitrogen, and insoluble nitrogen on a moisture-free basis are inversely related to the solids content. It is also apparent that, regardless of the solids content, the mg of nitrogen per g of fresh tissue is constant, within the experimental limits of error. These results can be interpreted that, for this variety of potato (Katahdin), all of the lots of tubers in this experiment stored essentially a constant amount of nitrogen and that the nitrogen did not vary appreciably throughout the storage period. The apparent inverse relationship between solids content and content of all forms of nitrogen on a moisture-free basis is the result of an increased content of some other dry matter constituent, presumably starch. Therefore, the nutritional value of a specific lot of potatoes, as measured by total, soluble, and insoluble nitrogen, is the same, whether it is from a fraction of high, intermediate, or low solids content. A dehydrated product produced from high-solids potatoes, however, would have a lower relative nitrogen value. Because there are known and unknown effects from environment and variety, care must be taken in generalizing on the relationship of specific gravity and nitrogen content until much more data have been obtained.

It is interesting that to be reconstituted, flakes produced from low-solids potatoes require a higher ratio of water than flakes from high-solids potatoes, Cording and Sullivan (1960). For these two products, the end result would approach the same nitrogen content per gram of reconstituted mashed potato.

The tables also indicate that, within a particular specific-gravity fraction, the relation of nitrogen level to actual solids content is more difficult to see. The reason is probably the relative insensitivity of the methods for determining the solids. It was for this reason, as well as to lessen the number of samples and to accentuate the differences due to specific gravity, that only the three specific-gravity fractions were studied. Close inspection of the data, however, indicates the same inverse relationship between solids and the *insoluble* nitrogen contents in all specific-gravity fractions.

The relationship of insoluble nitrogen to total nitrogen remains fairly constant, irrespective of solids content. The value of 38–40%, as presented, is corroborated by values previously reported (Chick and Slack, 1949).

The problem of sampling potatoes for specific-gravity studies is pointed up by the variation in the percentage of the sample in each fraction resulting from the brining operation. The variation, e.g., 3.68–25.32%

Table 5. The solids and nitrogen composition of low-specific-gravity fraction.

Month	% solids	% of sample	% total N (MFB)	mg total N/g fresh wt	% N in extract (MFB)	mg extractable N/g fresh wt	% insol N (MFB)	mg insol N/g fresh wt	Insol N / Total N (MFB)
Sep.	15.57	20.22	2.22	3.50	1.26	1.96	0.96	1.54	0.43
Oct.	14.32	12.36	2.32	3.34	1.39	1.99	0.93	1.35	0.40
Nov.	13.93	14.05	2.23	3.08	1.51	2.10	0.72	0.98	0.32
Dec.	14.50	3.68	2.34	3.39	1.40	2.04	0.94	1.35	0.40
Jan.	15.13	17.24	2.24	3.38	1.59	2.37	0.65	1.01	0.29
Feb.	15.48	25.32	2.21	3.42	1.34	2.07	0.87	1.35	0.39
Mar.	14.94	11.11	2.29	3.42	1.48	2.21	0.81	1.16	0.35
Apr.	14.91	23.84	2.26	3.37	1.43	2.14	0.83	1.23	0.37
May	15.31	22.64	2.23	3.28	1.38	2.12	0.85	1.16	0.38
June	14.67	12.52	2.43	3.56	1.54	2.27	0.89	1.29	0.37
Average	......	....	2.28	3.37	1.43	2.13	0.85	1.24	0.37

in the low-specific-gravity fraction (Table 5), results from the variation in the median specific gravity of the subsamples taken from storage for analysis, and is probably due to the inherent difficulty of selecting entirely representative samples from the original one-ton lot. It should be noted that this variation appears not to affect the over-all relation of nitrogen content to solids content, but the possibility exists that more accurate data could be obtained if this factor were eliminated. For this reason, future studies will be made on samples separated into the different specific-gravity levels before placement in storage.

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The Fatty Acids of Vegetables. II. Spinach[a, b]

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SUMMARY

The fatty acids in the extracted crude lipid of spinach were studied to determine changes during storage at −17.8°C in the blanched and untreated condition. It was further determined that the total free fatty acids increased in the unblanched samples during storage. Palmitic acid increased during storage, whereas the longer-chain fatty acids, particularly linolenir acid, decreased. A fatty acid containing 17 carbon atoms. *n*-heptadecanoic acid, was present in fair quantity.

The proximate composition of the lipid matter in spinach has been known for some time. The distribution of fatty acids was reported by Speer *et al.* (1929); Lee (1954) showed that spinach leaves held in storage at −17.8°C for relatively long periods undergo changes in the lipid material that are characterized by the development of acids and peroxides. Changes in the fatty acids in developing spinach leaves are of importance in studying the biochemistry of this vegetable. Furthermore, changes in fatty acid distribution could have considerable bearing on flavor changes in this vegetable during storage. The amount of lipid material present is small, but the lipids are likely to be important in flavor changes.

METHODS

Samples of spinach, Heavy Pack variety, were harvested at optimum market maturity from Experiment Station plots in 1951 and 1959. Following harvest, the stems were removed, and the leaves were washed. The samples in both years were divided into two parts. One part was placed raw in a 30-lb friction-lidded can, and frozen and stored as previously described. The 1951 material was removed from storage after 8 years and dried by lyophilization. The 1959 material was lyophilized immediately after freezing. In all cases the crude lipid was extracted from the dried samples, as described fully in a paper on peas (Lee and Mattick, 1961), which also described the separation of the several lipid fractions, liberation of the fatty acids, and the gas chromatographic equipment used for separation and identification of the fatty acids.

RESULTS AND DISCUSSION

The results are in Table 1. Table 2 lists the changes in fatty acids contained in the free fatty acids, neutral fats, and phospholipids, using as the starting point the figures in Table 1.

These data confirm an earlier report (Lee, 1954) that a relatively large quantity of free acids accumulate in unbalanced spinach during storage at −17.8°C. The data indicate that the blanching process attenuates the lipolytic enzyme systems of the spinach.

Compared with the blanched spinach as standard, the 1951 samples showed the following results. Palmitic acid showed a large increase, palmitoleic acid and *n*-heptadecanoic acids decreased, oleic and linoleic acids increased, and linolenic acid decreased drastically. In the 1959 samples, palmitic acid increased while all the others decreased, although, as one would expect, the decrease in linolenic acid was not great. This is doubtless due to the fact that the samples were fresh, but it does indicate that certain changes tend to start rather soon.

The data show that linolenic acid is lost from the phospholipid and neutral fat fractions. These losses cannot be explained by

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[b] Presented before the Twentieth Annual Meeting of the Institute of Food Technologists, May 24, 1960.

Table 1. Distribution of fatty acids in extracted lipid of spinach harvested in 2 years and analyzed in 1959 (mg fatty acids per 500 g spinach, dry-weight basis).

	1951						1959					
	Unblanched			Blanched			Unblanched			Blanched		
Fatty acids	FFA [a]	NF [b]	Ph [c]	FFA	NF	Ph	FFA	NF	Ph	FFA	NF	Ph
Caprylic	....	18	....	....	6	....	19	....	....	....	....	....
Pelargonic	0.4	....	....	1	....	....	2	....	....	1	....	....
Capric	10	13	....	2	6	....	28	....	109	8	....	....
Lauric	16	9	....	7	18	....	28	4	....	8	8	....
Dodecenoic	10	....	....	3	....	....	....	....	....	3	....	....
Myristic	51	40	30	5	41	12	23	23	....	6	12	10
Tetradecenoic	59	....	....	....	....	....	....	....	....	16	....	....
n-pentadecanoic	94	26	30	2	65	12	15	....	....	10	....	10
Palmitic	393	1304	1829	2	687	1378	38	730	1157	4	462	954
Palmitoleic	41	171	241	2	563	222	....	68	127	....	56	235
n-heptadecanoic	171	202	176	7	827	128	....	549	....	....	549	197
Oleic	104	391	392	....	252	298	....	117	104	....	219	182
Linoleic	314	606	603	61	423	881	....	218	654	....	295	819
Linolenic	774	1611	1729	30	2981	2878	....	2054	2387	....	2384	2386

[a] Free fatty acids.
[b] Neutral fat.
[c] Phospholipid.

the increase in the free fatty acid fraction, since they are not found in these quantities in this fraction. A possible explanation might be the conversion of the unsaturated longer-chain fatty acids and the saturated C_{17} acid to palmitic acid as the end product during storage at −17.8°C. The hydrogenation of unsaturated fatty acids in animal organisms has been demonstrated by Rittenberg and Schoenheimer (1937). An oxidation of the resulting saturated acid by an α and/or β oxidation pathway could result in palmitic acid (Stumpf and Bradbeer, 1959). This does not consider those acids present in small quantity. It was thought that they would not substantially alter the results.

It was recently shown in peas in this laboratory that palmitic acid increases while the longer-chain saturated and unsaturated acids decrease (Lee and Mattick, 1961). The phospholipid fraction appears to be the source predominantly affected, whereas in spinach both the phospholipid and the neutral fat fractions appear affected at the same rate.

In Table 2, 473 and 514 mg of fatty acid calculated as palmitic acid is not accounted for by the palmitic acid originally present plus conversion of the other fatty acids. The authors believe the discrepancy to be due to losses of soluble material in blanching. It is known that the blanching process will leach soluble substances from the product being blanched, thus changing the composition on the dry-weight basis.

The presence in fair quantity of the C_{17} fatty acid, *n*-heptadecanoic acid, is rather unusual, and of considerable interest.

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Table 2. Differences between blanched and unblanched spinach in distribution of fatty acids of lipid during storage at −17.8°C (acids calculated as weight of palmitic acid mg in 500 g dry weight).

Original fatty acid calculated to palmitic acid	Free fatty acid			Neutral fat			Phospholipid		
	UB [a]	B [b]	Diff [c]	UB	B	Diff	UB	B	Diff
Harvested 1951; stored 8 yr at −17.8°C; analyzed 1959									
Caprylic	....	....	....	10	3	7	....	....	....
Pelargonic	0.2	0.6	....	....	....	....	....	....	....
Capric	7	1	6	9	4	5	....	....	....
Lauric	12	5	7	7	14	−7	....	....	....
Dodecenoic	8	2	6	....	....	....	....	....	....
Myristic	45	4	41	36	37	−1	27	11	16
Tetradecenoic	52	....	52	....	....	....	....	....	....
Pentadecanoic	89	2	87	25	61	−36	28	11	17
Palmitic	393	2	392	1304	687	656	1829	1378	451
Palmitoleic	41	2	49	170	559	−389	239	220	19
Heptadecanoic	180	7	172	213	872	−659	186	167	19
Oleic	115	....	115	431	278	153	432	328	104
Linoleic	343	68	275	663	463	200	660	964	−304
Linolenic	841	33	808	1750	3237	−1487	1878	3125	−1247
Harvested 1959; frozen and lyophilized; analyzed immediately									
Caprylic	11	....	11	....	....	....	....	....	....
Pelargonic	1	1	....	....	....	....	....	....	....
Capric	19	5	14	....	....	....	73	....	73
Lauric	22	6	16	3	6	−3	....	....	....
Dodecenoic	....	2	−2	....	....	....	....	....	....
Myristic	20	5	15	20	11	9	....	9	−9
Tetradecenoic	....	14	−14	....	....	....	....	....	....
Pentadecanoic	14	9	5	....	....	....	....	9	−9
Palmitic	38	4	34	730	462	268	1157	954	203
Palmitoleic	....	....	....	67	56	11	126	233	−107
Heptadecanoic	....	....	....	579	579	....	....	208	−208
Oleic	....	....	....	129	241	−112	115	200	−85
Linoleic	....	....	....	238	323	−85	715	895	−180
Linolenic	....	....	....	2231	2591	−360	2592	2591	1

[a] Unblanched.
[b] Blanched.
[c] Difference is equal to the unblanched minus the blanched.

Study of a Lipohydroperoxide Breakdown Factor in Soy Extracts [a]

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SUMMARY

The rapid decrease in linoleic acid hydroperoxides observed with relatively high levels of soy flour extract was investigated. The hydroperoxide breakdown factor can be eliminated by high-temperature short-time treatment of soy extracts. The addition of KCN to reaction mixtures caused a partial inhibition of the breakdown factor activity. Variations in hydroperoxide breakdown activity with variations in pH and reaction time were studied. A possible optimum at pH 8–9 was indicated. Qualitative evidence for the presence of a lipohydroperoxidase was obtained with well-known reagents that produce a specific color reaction with peroxidases.

In studying the reactivity of legume lipoxidase, Dillard *et al.* (1961) noted an extremely rapid hydroperoxide production, followed by an equally rapid decomposition, when they used a comparatively high level of enzyme extract (0.2 ml). This unusual effect is of great interest since it suggests the presence of an enzymatic mechanism, perhaps a peroxidase, that might shed new light on the reason for the existence of lipoxidase in legumes.

Privett *et al.* (1955) observed that the various quantities of secondary products produced by lipoxidase could be decreased by using a low concentration of the enzyme and a low temperature of oxidation. They stated that non-hydroperoxide material may form to some extent by hydroperoxide decomposition during and subsequent to oxidation, but also that various evidence indicated that secondary products were formed directly during the oxidation. Dillard *et al.* (1961) also showed that hydroperoxide breakdown was not evident when very low levels of enzyme activity were studied.

Blain and Styles (1959) recently reported that soya extracts have a "lipoperoxidase" activity similar to that of cytochrome c in that preformed linoleate peroxide was used by both to bleach β-carotene. They found that buffered extracts possessed greater peroxidase activity than did water extracts.

The following study was undertaken to ascertain whether the rapid decrease in hydroperoxide observed by Dillard *et al.* (1961) was due to an enzymatic destruction of the products of the lipoxidase action.

MATERIALS AND METHODS

Substrate and reaction mixture. Substrates from purified linoleic acid and buffers were prepared as described by Koch *et al.* (1958). Linoleic acid was used as the sole substrate throughout this investigation. Both the enzyme extraction procedure and the method of Sumner and Somers (1947) for the measurement of hydroperoxides as reported by Koch *et al.* (1958) were used in this study.

Enzyme extraction. The water-soluble extract of defatted soy flour (80 g/800 ml) was used as the primary source of enzymes after removal of precipitate produced by addition of 50 ml aqueous $CaCl_2$ (64 mg/ml) to the extract.

Enzyme inactivation. *Heat.* Five-ml portions of a $CaCl_2$ supernatant were pipetted into 25-ml Erlenmeyer flasks, heated for varying periods in a

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hot-water bath at 84°, cooled under tap water immediately, and filtered through Whatman No. 4 filter paper. The resultant filtrates were assayed for lipoxidase activity.

Chemical. The reaction mixture consisted of 100 ml pH 7.5 phosphate buffer, no substrate, 0.5 ml of 1*M* KCN, and sufficient 1*N* NaOH to buffer the solution at pH 8.0 at 20°C. One-half ml of the $CaCl_2$ supernate of soyflour extract was added and held for 2 min. Then, 1.0 ml of linoleic acid (10 mg/ml) was added. Two-ml portions were removed at increasing time intervals and assayed for hydroperoxides. Controls included a reaction mixture in which 0.5 ml of 1*M* KCl was substituted for the cyanide, and one that contained only buffer, enzyme, and substrate. The final pH of the several reaction mixtures was read on a Beckman pH meter after completion of the experiment, and was found not to change.

pH variations. To 100 ml of pH 7.0 phosphate buffer, sufficient 1*N* NaOH was added to adjust the pH of several buffer solutions to 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, and 10.5. pH curves for 0.05 ml, 0.1 ml, and 1.0 ml enzyme were determined at 20°C with linoleic acid (10 mg/ml). Final pH after 16-min reaction time was determined after each run.

Qualitative determination of peroxidase. Peroxidase activity in reaction mixtures containing 1.0 ml fatty acid substrate (10 mg/ml), enzyme extract, and 5.0 ml buffer at pH 8.1, was determined qualitatively. Reagent grade guaiacol and *o*-phenylenediamine were prepared. The guaiacol reagent (0.2 ml of a 1% solution in 95% ethanol) was added to reaction mixtures containing soy extract, and linoleic acid at pH 8.0 after a 16-min reaction time at 20°C. The phenylenediamine reagent (1 ml of a 0.1% aqueous solution) was added to a second identical reaction mixture after a 16-min reaction time.

The experimental design followed in this investigation included: 1) determination of the effect of increasing enzyme extract on level and rate of hydroperoxide production and breakdown, 2) attempt to separate lipoxidase and hydroperoxide breakdown factor by ammonium sulfate fractionation, 3) differential enzyme inactivation by heat, 4) use of KCN, a known peroxidase inhibitor, to determine its effect on hydroperoxide formation and disappearance, 5) presentation of data indicating peroxidase stability, and 6) direct qualitative evidence for peroxidase action obtained by a method similar to that of Vetter *et al.* (1958).

RESULTS AND DISCUSSION

Repeated attempts to obtain a highly active enzyme preparation from defatted soy flour from a new crop of soybeans was apparently unsuccessful. This was in contradiction to earlier work by one of the authors (Dillard *et al.*, 1961; Koch *et al.*, 1958). An absorbance greater than 0.600 could not be obtained even after taking precaution to extract the soy flour in an ice bath, to increase the levels of flour extracted, and to increase the levels of extract added to the substrate. It will be observed from Fig. 1 that apparent low lipoxidase activity was obtained, especially at the highest enzyme level. A loss of peroxide occurred after shorter reaction periods for each increase in enzyme extract above 0.2 ml.

A similar effect was observed when a dialyzed ammonium-sulphate-precipitated fraction of a soy-flour extract (after Koch *et al.* 1958) was assayed for activity against linoleic acid at pH 8.0. Table 1 shows the activity of this fraction with time. Although maximum activity was higher obtained with this more purified fraction, a rapid decrease in reaction product still occurred.

The decreased activities found here confirmed the observations of Dillard *et al.* (1961) and suggested that an enzyme contaminant was present in the extracts.

A number of enzymes associated with hydroperoxide decomposition in plant sources have been purified, crystallized, and carefully characterized during the last sev-

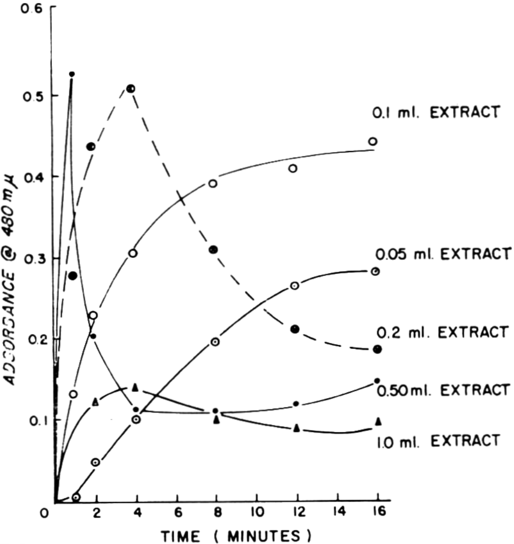

Fig. 1. The lipoxidase activity of increasing levels of soy extract. Reaction mixture: 3.5 × 10⁻ moles linoleic acid, pH 8.1, and 20°C.

Table 1. Activity of an ammonium-sulfate-fractionated sample from a soy extract.

Time (min)	Absorbance (480 mμ)
1.0	0.426
2.0	0.891
3.0	0.961
4.0	0.626
8.0	0.533
16.0	0.241
32.0	0.150

Reaction mixture: 3.5×10^{-4} moles of linoleic acid (10 mg/ml), pH 8.0, 0.05 ml enzyme fraction, and 20°C.

eral decades (Lardy, 1949). They have been classified as peroxidases. It was highly probable that an enzyme contaminant of this class might be responsible for the aforementioned peculiarities in peroxide values. To examine this probability, a number of studies were made that affect this type of enzyme. The results support the hypothesis that peroxidase was responsible for hydroperoxide breakdown.

Heat inactivation of the hydroperoxide-decomposing factor. Heat treatment of $CaCl_2$ supernatants prepared from freshly defatted soybean flour extracts was attempted according to the procedure described under materials and methods. Heating 37 sec at 84° yielded the greatest removal of hydroperoxide-decomposing factor with the least destruction of lipoxidase activity (Fig. 2).

The heat treatment was sufficient to yield a striking increase in lipoxidase activity over crude extracts of the soy flour. Under the specified heating conditions, hydroperoxide decomposition was still readily apparent after 30- and 35-second heating times. A 45-second heating period rendered the crude extract completely inactive to linoleic acid. The hydroperoxide-decomposing factor demonstrated a very sharply defined inactivation time under the above conditions. The inactivation time was only a few seconds longer than the time required for macroscopic observation of protein coagulation and denaturation.

At this point, the sharply defined heat inactivation could be explained either by the fact that an activation of the lipoxidase had occurred or by the more plausible explanation that a peroxidase inactivation had been effected. With proper heating conditions this subsequent phenomenon of increased lipoxidase activity was observed repeatedly, even on old soybean flour extracts that had been refrigerated at 4° for longer than 1 week. That the differential inactivation in these studies was not complete is also illustrated in Fig. 2, which shows a slow decrease

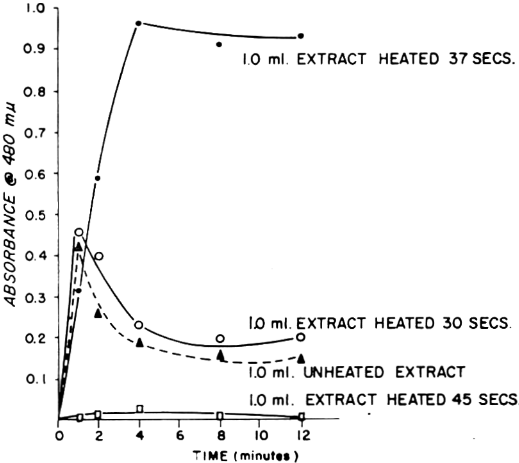

Fig. 2. The effect of heating 5.0 ml of soy extract at 84°C for different periods. Reaction mixture: 3.5×10^{-4} moles of linoleic acid, pH 8.1, and 20°C.

in peroxide after the maximum value was reached for the sample heated 37 sec.

Chemical inactivation of hydroperoxide-destroying factor. Having postulated the presence of a peroxidase, its inactivation by a direct chemical method was ventured. The effect of KCN on a soy-flour extract is presented in Fig. 3. The results demonstrate that the action of the hydroperoxide-destroying factor can be somewhat delayed by $5 \times 10^{-3} M$ KCN. Since it is known that cyanide has no effect on lipoxidase, it is assumed that the observed difference was due to reaction with a peroxidase. Fig. 3 also shows that the higher peroxide values are not due to a salt effect.

Since $5 \times 10^{-3} M$ KCN in the reaction mixture had only a slight effect on hydroper-

oxide production, it was of interest to investigate the response to a higher level of KCN. However, since KCN is a strong base, it was necessary to carry out the studies at pH 10.5. A comparison of the results in Table 2 shows that after a 1-min

Table 2. The effect of KCN on hydroperoxide production.

Time (min)	Absorbance @ 480 mμ	
	Control [a]	KCN [b]
1	0.200	1.28
2	0.417	1.36
4	0.800	1.27
8	1.00	1.11

Reaction mixture: [a] 1.0 ml enzyme extract; 3.5×10^{-4} moles linoleic acid; pH 10.1, 20°C. [b] 0.5 ml enzyme extract; 3.5×10^{-4} linoleic acid, pH 10.5, 20°C; and 5 ml 1*M* KCN. Enzyme extract added to reaction mixture 5 min prior to addition of substrate. Zero time when substrate added.

reaction time when twice the amount of extract was used in the control, over six times the amount of hydroperoxide was produced when KCN was present at a concentration of $50 \times 10^{-3} M$. The results in Table 2 also indicate that the hydroperoxide breakdown factor, although still active, was not as effective as it was at pH 8.0 (Fig. 1).

Further evidence for a possible pH optimum near 8.0 (Fig. 5) is discussed later.

Peroxidase activity in a soy extract stored 7 days at 4° C. The soy extract used to obtain the data in Fig. 1 was held 7 days at 4°C, after which there was either a loss of lipoxidase activity or an increase in the activity of the hydroperoxide breakdown factor. The use of KCN ($50 \times 10^{-3} M$) under experimental conditions given in Table 2 resulted in the production of a hydroperoxide value similar to that presented in Table 2 and indicated that there was no apparent loss in lipoxidase activity, so that the hydroperoxide breakdown factor activity must have increased. To demonstrate further the presence of the latter activity, 0.5 ml of the stored extract was added to 0.1 ml of fresh extract. The results (Table 3) indicate that the hydroperoxide breakdown factor was more active in the extract after holding at 4°C than it was when the fresh extract was used to obtain the 0.5 ml curve in Fig. 1.

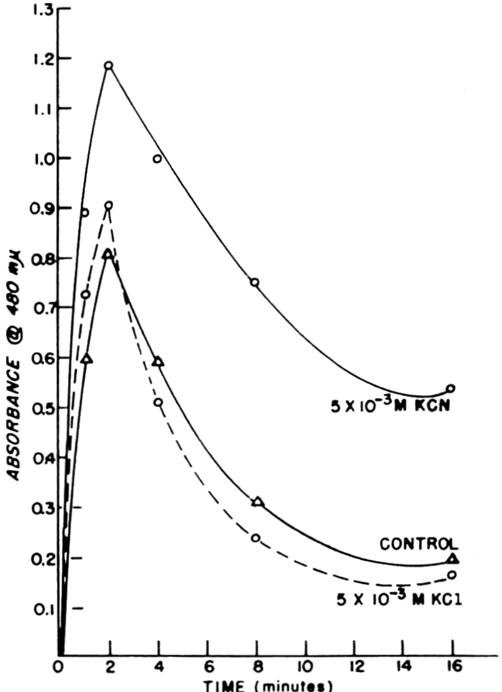

Fig. 3. The effect of KCN on the lipoxidase activity of soy extracts. Enzyme extract added to reaction mixtures 2 min before substrate. Reaction mixture: 3.5×10^{-4} moles of linoleic acid, 0.5 ml soybean extract, pH 8.2, and 20°C.

Effect of variations in pH and reaction time. The observation that levels of hydroperoxide were higher at pH 10.1 even in the absence of KCN (Table 2) led to investigation of the effect of pH on the hydro-

Table 3. Effect of the addition of stored soybean flour extract to fresh extract.

Enzyme extract held 7 days at 4°C	None	0.5 ml	0.5 ml
Fresh enzyme extract	0.1 ml	0.1 ml	None
Time (min)	Absorbance @ 480 mμ		
1.0	0.132	0.205	0.196
2.0	0.233	0.154	0.187
4.0	0.315	0.128	0.158
8.0	0.442	0.122	0.143

Reaction mixture: 3.5×10^{-4} mole linoleic acid, pH 8.0, and 20°C.

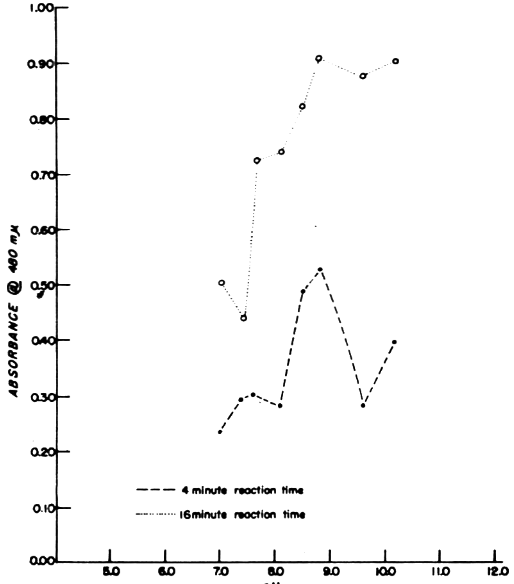

Fig. 4. The lipoxidase activity of soy extracts at two different time intervals and at different pH. Reaction mixture: 3.5×10^{-4} moles linoleic acid, 5.0 ml of phosphate buffer, pH adjusted with 1*N* NaOH, 20°C, and 0.05 ml soybean extract.

peroxide breakdown factor at high and low levels of enzyme extract. As shown in Figs. 4 and 5, time of reaction as well as pH have a pronounced effect on the production of hydroperoxides. The data in Fig. 4 illustrate that at a low enzyme concentration there was a positive correlation between hydroperoxide production and time of reaction. A pH optimum of 8.5–8.8 was observed for crude enzyme extract. The pH curve was more distinct for the shorter reaction time.

A completely different set of curves was obtained when a high level of crude enzyme extract was used (Fig. 5). A negative correlation between hydroperoxide production and reaction time was observed below pH 10.0. This reversal in correlation indicates that the concentration of the hydroperoxide breakdown factor was such that it was not effective when low levels of enzyme extract were used in the reaction mixture. Also, the dip in the pH curves (Fig. 5) between pH 8 and 9 and the considerably higher hydroperoxide content in this pH range for the 4-min curve indicate a possible pH optimum for the hydroperoxide breakdown factor.

Qualitative determination of peroxidase activity. To demonstrate further the presence in the soy extracts of a peroxidase whose existence was recently suggested by Blain and Styles (1959), a method for direct measure of its activity was used. Two different substrates, known to be reactive with horseradish peroxidase, were added to the mixtures as described under materials and methods. Guaiacol (Lardy, 1949; Sumner, 1943) or o-phenylenediamine (Sumner and Somers, 1947) was added, and a distinct orange or yellow developed in the respective flasks, indicating the presence of a peroxidase. The heat-treated soy extract (see above) gave a negative test for peroxidase with these reagents. However, the KCN-treated extracts showed a slight peroxidase activity remaining even in the presence of relatively large amounts of KCN. Similar

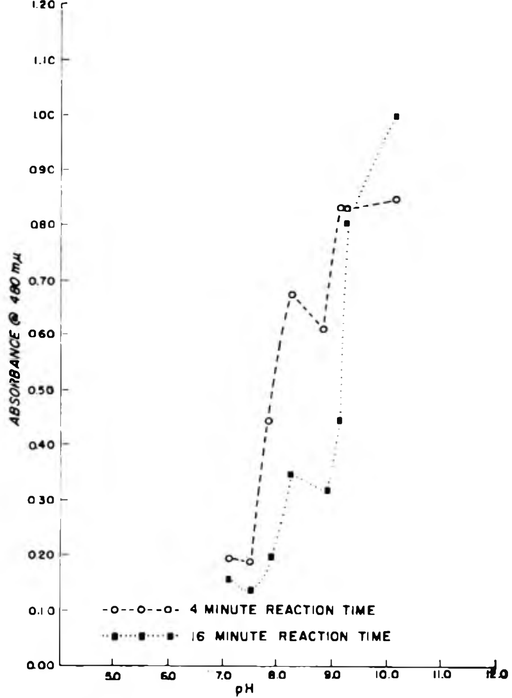

Fig. 5. The lipoxidase activity of soy extracts at two different time intervals and at different pH. Reaction mixture: 3.5×10^{-4} moles of linoleic acid, 5.0 ml phosphate buffer, pH adjusted with 1*N* NaOH, 20°C, and 1.0 ml soybean extract.

color reactions were observed when 1.0 ml of 30% H_2O_2 was added to the reaction mixtures in place of the linoleic acid. Color development was apparent only when high levels of enzyme extract (1.0–2.0 ml) were used.

Tests of navy bean and split green peas under identical conditions indicated that both products contained lipohydroperoxide breakdown activity. However, it appeared that the peas and soybeans had much greater lipohydroperoxidase activity than navy beans.

These results give strong presumptive evidence that the rapid decrease in hydroperoxide, reported by Dillard *et al.* (1961) when relatively high levels of fresh soy extract are reacted with linoleic acid, was due to the presence of a peroxidase. This enzyme(s) is apparently present in the soy extract at a much lower level of activity than the lipoxidase enzymes. However, the peroxidase appears to become more active by holding at low temperature for short periods. The evidence for a reaction sequence between lipoxidase and peroxidases points to the possible existence of a hitherto unknown mechanism for the action of lipoxidases in legumes.

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Precipitation of Food Gums by Thiazine, Oxazine, Azine and Other Cationic Dyes: Specificity of the Methylene Blue Carrageenan Reaction

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SUMMARY

Several dyes of the thiazine, azine, azo and other classes were mixed with carrageenan, furcellaran, fucoidan and other hydrocolloids and the nature of the precipitates formed was carefully observed. The results obtained indicate that the antigen-antibody-like specificity ascribed to the methylene blue-carrageenan reaction is much less rigid than previously thought.

Dyes bearing the thiazine, phenazine, azine, oxazine or azo core structure will give the typical methylene blue-carrageenan stringy precipitate when mixed with the hydrocolloids mentioned above. The sulfated polygalactose moiety is an essential for the formation of such a precipitate. Salts, at concentrations above certain threshold levels, inhibit formation of the stringy precipitate and, at low pH levels of the medium, the strings are considerably shortened. It is postulated that formation of the stringy precipitate results from the tendency of the linear macromolecules of carrageenan to agglomerate into fibers on precipitation from solutions.

Carrageenan, a sulfated plant hydrocolloid used widely as a stabilizer in the food industry, gives, when mixed with methylene blue, a characteristic stringy precipitate that is used as a confirmatory test (Ewart and Chapman, 1952; Jacobs, 1939; Assoc. Off. Agr. Chemists, 1955). Ewe (1930) published the first report on this unique precipitate. Of several dyes he tested, only methylene blue gave the stringy precipitate, and of the gums he tested only carrageenan gave this reaction. No further investigation on this problem has been reported. Stoloff and Silva (1957) considered this specificity to be akin to the antigen-antibody reaction. Stoloff (1954, 1959) suggested that a study of the relative structures of precipitants and non-precipitants of carrageenan might shed some light on the carrageenan configuration, since positively charged macromolecules do not necessarily precipitate the polyanionic phytocolloid. No systematic study has been made, however, of the reactions of carrageenans or polysaccharides related to Irish moss extractive with dyes related and unrelated to methylene blue.

Since methylene blue, an important thiazine dye, is structurally closely related to the oxazine and azine dyes, it was suspected that the latter classes of dyes, and probably others with related core structure (Table 1) may also give the characteristic precipitates with carrageenan. Similarly, carrageenan is structurally related to such sulfated polysaccharides as furcellaran, fucoidan, iridophycan, funoran, hypnean, Eucheuma extract, gums from lesser-known red algae (Clingman *et al.*, 1957; Jones and Smith, 1949; Nunn and von Holdt, 1957; Painter, 1960), and the supposedly lightly sulfated polysaccharide agar. The other hydrocolloids, then, could possibly give the typical precipitate with methylene blue.

No fundamental explanations have ever been advanced for the production of a stringy precipitate when methylene blue is mixed with carrageenan or polysaccharides related to Irish moss extractives. On the structural level, two possibilities exist. Firstly, some peculiar characteristic of car-

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Table 1. Plant hydrocolloids that gave stringy precipitates with methylene blue and some other cationic dyes.

Dye	Classification and core structure	Gums giving reaction		
		Carrageenan and its fractions	Iridophycan	Furcellaran
Methylene blue Toluidine blue Methylene green Thionin Thioflavin T	Thiazine	+	+	+
Safranine	Phenazine	+	+	±
Neutral red	Azine (Amidoazine)	+	+	+
Cresyl violet acetate Meldola blue Brilliant cresyl blue	Oxazine	+	+	+
Bismarck brown	Azo	+	+	+
Pyronin	Xanthene	+	+	+

rageenan and carrageenan-like hydrocolloids could be involved. Secondly, some special configuration of the dye could be necessary for the unique reaction. Alternately, there could exist the necessity of some inorganic (major or minor) element to cement the unusual architecture of the precipitate. This article reports on attempts to elucidate the basic nature of this reaction and some of the conditions governing formation of the precipitate. The data presented indicate that development of the stringy precipitate is dependent on the structure of both the interactants, i.e., the dye and the hydrocolloid; that string formation is inhibited by salts when they are present above certain threshold levels; and that the strings are considerably shortened at low pH levels of the medium.

EXPERIMENTAL PROCEDURE

Materials: Tables 1-3 list the various dyes used, some of their major structural characteristics, and their interactions with several gums.

All inorganic salts, of c.p. grade, were used without further purification. They were dissolved in distilled water, and the strength of the stock solution was 0.1M.

Preparation of Gum Samples: Except where extraction had to be done, 0.25% gum samples were prepared by dusting 0.25 g of the particular gum onto distilled water. Complete solution was achieved by heating, with constant stirring. Finally, the total volume was made up to 100 ml with distilled water. When necessary, further dilutions were done with distilled water.

Preparation of Dialyzed Gum Solutions: Five-tenths per cent solutions of the gums were placed in 36/32 dialysis membranes and dialyzed against distilled water for 36 hours with six changes of water.

Preparation of Dye Solutions: All dye solutions were 0.1-0.5% in concentration and made up in distilled water.

Equipment: The equipment was volumetric pipettes and 150 × 15-mm Pyrex test tubes.

Procedure: A. *Nature of the Precipitates Formed.* Five ml of the gum samples were placed in a test tube, and 1 ml of the particular dye added. The mixture was allowed to stand for 5 minutes, and the nature of the precipitate was observed visually. Since the methylene blue-carrageenan precipitate has been well characterized and is readily reproduced, this was taken as the standard. Other types of precipitates were recorded as granular or flaky. Vigorous agitation of the reaction tubes should be avoided, since this may give misleading results.

B. *Influence of pH on Formation of the Stringy Precipitate.*—Since the reaction between the dye and the gum is primarily ionic in nature, the pH of the medium would influence the degree of interaction and, possibly, the degree of stringiness. To assess the influence of pH on the latter, phosphate buffers were made up of the pH levels indicated in Table 4. The carrageenan-methylene blue interaction was allowed to occur in such buffers and the degree of stringiness was observed at each pH level. For the two lowest pH levels, H_3PO_4 was used instead of the sodium phosphate buffer.

C. *Influence of Salts on Formation of the Stringy Precipitate.*—Increasing amounts of the salts listed in Table 5 were added to 5 ml of the carrageenan solutions, and the tubes vigorously shaken. One ml of the methylene blue solution was then added, the contents were mixed, and the precipitate formed was observed visually. The final volume of each reaction tube was 10 ml in all cases, distilled water being included when necessary.

To establish the concentration of a particular salt that would just inhibit formation of the typical strings, an arbitrary line was set up at a visual point where very short strings began to assume the configuration of small granules or flakes. This point is conveniently dubbed *"the threshold level."*

RESULTS AND DISCUSSION

The results in Tables 1–5 clearly indicate that methylene blue and other dyes listed in Table 1 produce an unequivocally stringy precipitate when the polygalactose and/or the 3,6-anhydrogalactose structure is present. Polymers of other sugars do not give the characteristic precipitate, as evidenced by failure of polyglucose sulfate, dextran sulfate, and fucoidan to do so.

Ester sulfate in the polysaccharide is apparently essential to formation of the stringy precipitate since both polygalacturonic acid and pectin failed to give the typical precipitate. In addition, the degree of esterification greatly influences formation of the stringy precipitate. This is substantiated by the failure of agar, a supposedly lightly sulfated polysaccharide, to produce stringiness. Thus, the necessary esterification must be on the order of that found in carrageenan (Sea-Kem Extracts, 1957; Smith and Montgomery, 1959), iridophycan (Hassid, 1933; Smith and Montgomery, 1959), or furcellaran (Schachat and Glicksman, 1959a, b;

Table 2. Plant hydrocolloids and other compounds that did not give a stringy precipitate with methylene blue and other dyes.

Hydrocolloids or other compounds	Precipitates obtained with:							
	Methylene blue	Neutral red	Toluidine blue	Thionin	Acridine orange	Meldola blue	Cresyl violet acetate	Janus green
Hypnean	Granular	Granular	Granular	Granular	Granular	Granular	Granular	Granular
Chondroitin sulfate	Granular	Granular	Granular	Granular	Granular	Granular	Granular	Granular
Polygalacturonic acid	Flaky	Flaky	Flaky	Granular	Flaky	Flaky	Flaky	Flaky
Fucoidan	Granular	Granular	Granular	Granular	Granular	Granular	Granular	Granular
Dextran sulfate	Granular	Granular	Granular	Granular	Granular	Granular	Granular	Granular
Sodium polyglucose sulfate	....	....	....	....	....	....	....	....
Quinine sulfate	....	....	....	....	....	....	....	....
Cinchonine sulfate	....	....	....	....	....	....	....	....
Cellulose gum	Granular	Granular	Granular	Granular	Granular	Granular	Granular	Granular
Pectin	Granular w/few strings	Granular	Granular	Granular	Granular	Granular	Granular	Granular
Agar	Flaky	Flaky	Flakes, some cotton-like	Flakes, some cotton-like	Flaky	Flaky	Flaky	Flakes, cotton-like
Gum acacia	Granular w/few strings	Granular	Granular	Granular	Granular	Granular	Granular	Granular

Smith and Montgomery, 1959). Additional evidence on the specificity of the saccharine portion of the molecule is the failure of the sulfated mucopolysaccharide, chondroitin sulfate, to give a stringy precipitate. This, however, could indicate that a stringy precipitate requires that a high molecular weight ester sulfate be mixed with methylene blue or one of the dyes listed in Table 1.

Both lambda- and kappa-carrageenan gave the characteristic stringy precipitate when mixed with methylene blue. Thus, either structure will be adequate when present in polysaccharides at a sufficient level. However, since lambda-carrageenan gives the stringy precipitate with methylene blue, this apparently indicates that the 3,6-anhydrogalactose moiety is sufficient for, though not essential to, formation of the stringy precipitate.

Based on the carrageenan-methylene blue precipitate, the stringiness produced on reaction of the gums with methylene blue (Table 1) may be arranged in order of similarity as follows: 1) methylene blue-carrageenan; 2) methylene blue-iridophycan; and 3) methylene blue-furcellaran. Of this group, the methylene blue-furcellaran strings are the most fragile. On shaking, they readily fragmentize into lengths so short that they are on occasion difficult to recognize as strings. In addition, the age and concentration of the gum solutions, especially in the case of furcellaran, will apparently affect the reproducibility of the characteristic methylene blue-carrageenan-like precipitate.

On the basis of chemical similarities, hypnean would be expected to react like carrageenan. Failure in this case may be partially attributed to the extraction and purification procedures. Secondly, although considered to be similar to carrageenan and having corresponding kappa and lambda fractions, hypnean is a substance about whose chemical structure little is known (Smith and Montgomery, 1959). Extracts of *Gigartina acicularis, Gigartina pistillata, Eucheuma cottonii*, and *Eucheuma spinosum* gave results similar to those obtained with carrageenan.

With respect to the molecular configuration of the dye, the evidence is strong that production of a stringy precipitate depends on the presence of the thiazine, azine, azo, or oxazine structure. All dyes with these "core structures" (see Table 1) gave stringy precipitates with the gums listed. Further-

Table 3. Dyes that did not give stringy precipitates with gums listed in Table 1.

Dye	Classification of dye	Nature of precipitate obtained
Basic fuchsin	Triaminotriphenylmethane (rosaniline)	Flaky
Nigrosin	Induline	No visible reaction
Rhodamine	Rhodamine	No visible reaction
Crystal violet	Triaminotriphenylmethane (rosaniline)	Granular or Flaky

Table 3-A. Dyes that gave very slight or doubtful stringy precipitates with gums listed in Table 1.

Dye	Classification of dye	Nature of precipitate obtained
Acridine yellow	Acridine	Flaky w/few strings
Acridine orange	Acridine	Flaky/w strings
Methyl violet 2B	Triaminotriphenylmethane (rosaniline)	Flaky w/few strings

Table 4. Influence of pH on the nature of the precipitate formed by reacting carrageenan methylene blue and other dyes.

Dye	pH						
	1.35	2.42	4.75	7.0	7.6	8.9	10.0
Methylene blue	Shortened strings	Shortened strings	Stringy	Stringy	Stringy	Stringy	Stringy
Safranine O	Flaky w/few strings	Flaky w/few strings	Flaky w/some strings	Mixture of flakes and strings	Mixture of flakes and strings	Mixture of flakes and strings	Mixture of flakes and strings
Crystal violet	Granular	Granular	Granular	Granular	Granular	Granular	Granular

more, as seen from Table 3, basic fuchsin, a triaminotriphenylmethane dye, failed to produce the characteristic strings whereas methyl violet, a dye of the same class, gave a doubtful reaction. Still further evidence is the slight or doubtful stringy precipitates with the acridine dyes (Table 3). In this case the indeterminate reaction was probably caused by the close similarity of the acridine, azine, oxazine, and thiazine structures. The substitution of a carbon atom for nitrogen, oxygen, or sulfur could, among other things, account for the modified reaction. Note should be taken also of the fact that pyronin, a dye of the xanthene class to which acridine is closely related, gave stringy precipitates (Table 1). In this case, however, the strings were inclined to assume a "cottony" characteristic. If compared with the oxazines, it is again tempting to conclude that substitution of carbon for nitrogen modifies the reaction.

Since stringiness cannot be assessed quantitatively, it is difficult, if not impossible, to discuss any gradients that are caused by substituting groups on the "core" structure of the dyes listed in Tables 1–3. However, certain probabilities can be listed. Safranine, a phenazine dye, consistently gave strings relatively less outstanding than did the thiazine dyes or neutral red, an azine dye. This indicates that the attachment of a phenyl group to one of the nitrogens modifies the reaction. This conclusion is fortified by the failure to obtain stringy precipitates with nigrosin, an induline dye (a sub-class of the azines) that is more highly phenylated than safranine (Table 3). A similar influence of phenylation is seen by comparing the results for rhodamine B with those for pyronin. Both dyes are derivatives of xanthene. However, the rhodamines are similar to the pyronines, except that they contain another benzene ring with a carboxyl group in the ortho position.

From Table 4 it is evident that the pH of the reacting medium influences the reaction because, at low pH levels, the strings are considerably shortened. Since the interaction is ionic in nature, the reaction was impeded but not completely inhibited, and aggregation of the carrageenan-methylene blue precipitate into strings was evidently greatly minimized at the lower pH levels.

In every case, high concentrations of the salts tested inhibited formation of the characteristic stringy methylene blue-carrageenan precipitate. The data recorded in Table 5 indicate that the interference of added salts on formation of the stringy precipitate becomes more drastic as the valence of the cation increases. The "threshold levels" required to inhibit the stringiness of the carrageenan-methylene blue precipitate decreased on the order 0.1 : 0.01 : 0.001 in going from the monovalent to the divalent to the trivalent cations. The higher threshold level for $CrCl_3$ may be due to the fact that the Cr is present as the divalent complex $[CrCl(H_2O_5]^{++}$.

The stringy nature of the carrageenan-methylene blue precipitate probably manifests the property of the linear macromolecules of carrageenan to agglomerate into fibers on precipitation from solutions. Attention is directed to the fact that carrageenan forms a rather stringy precipitate on reaction with quaternary ammonium salts.

One common characteristic of all these complexes is their insolubility in aqueous solutions. The quaternary ammonium complexes, however, are more cottony than the distinct thread-like forms seen in the dye reactions. Thus, the phenomena are not identical and can be readily distinguished. More or less stringy precipitates are also formed by carrageenan on precipitation with alcohol and 5% ferric chloride (Assoc. Off. Agr. Chemists, 1955).

The data indicate that the antigen-antibody-like specificity ascribed to the methylene blue-carrageenan reaction (Stoloff, 1959) is much less restricted than reported. Production of the stringy precipitate is determined by the molecular configurations of both the gum and the dyes, the proximity of ester sulfate groups in the polysaccharide, and, apparently, the molecular weight of the polymers. The similarities of the precipitates obtained with the gums listed in Table 1 parallel the established structural similarities elucidated by chemical and physical methods (Jones and Smith, 1949; Smith and Montgomery, 1959). Structural dissimilarities in the case of agar are also reflected. Thus, such studies afford simple techniques with which to supplement findings arrived at by more elaborate means, an approach that has been exploited in protein chemistry (Fraenkel-Conrat and Cooper, 1944) and carbohydrate histochemistry (Mowry, 1956).

Table 5. Effect of salts on the formation of the methylene blue-carrageenan precipitate.

Monovalent cations	Salt used	Threshold level of salt (M)
K^+	KCl	0.17
Li^+	LiCl	0.20
Na^+	NaCl	0.28
NH_4^+	NH_4Cl	0.17
Divalent cations	**Salt used**	
Ba^{++}	$CaCl_2$	0.02
Ca^{++}	$CaCl_2$	0.05
Cd^{++}	$CdCl_2$	0.04
Hg^{++}	$HgCl_2$	0.02
Mg^{++}	$MgCl_2$	0.08
Mn^{++}	$MnCl_2$	0.10
Ni^{++}	$NiCl_2$	0.02
Sr^{++}	$SrCl_2$	0.02
Zn^{++}	$ZnCl_2$	0.05
Co^{++}	$CoCl_2$	0.03
Cu^{++}	$CuCl_2$	0.03
Trivalent cations	**Salt used**	
Al^{+++}	$AlCl_3$	0.002
Cr^{+++}	$CrCl_3$	0.02
Fe^{+++}	$FeCl_3$	0.001

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Changes in Soybean Lipids During Tempeh Fermentation[a, b]

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SUMMARY

Changes in the lipids of soybeans brought about by *Rhizopus oryzae* during the production of tempeh were studied. The mold possesses strong lipase activity and caused the hydrolysis of over one-third of the neutral fat of the soybean during the three-day fermentation. The fatty acid composition of soybean tempeh was compared with that of cooked soybeans by vapor-phase chromatography of the methyl esters. The neutral fat was composed of palmitic, stearic, oleic, linoleic, and linolenic acids, with linoleic acid predominating. These acids were liberated during fermentation in roughly the same proportions found in soybeans after heating 90 min at 100°C. During the most active mold growth, proportionately higher levels of palmitic acid were found, and the level of linoleic acid was somewhat lower. Except for the depletion of some 40% of the linolenic acid in the later stages of the fermentation, there apparently was no preferential utilization of any fatty acid.

Tempeh kedelee is a fermented soybean food product consumed widely in Indonesia and some other sections of the Far East. Cooked soybeans are inoculated with spores of *Rhizopus oryzae* and allowed to ferment for 1–3 days. The resulting product, digested considerably more readily than cooked soybeans, plays an important dietary role in Indonesia (Van Veen and Schaefer, 1950).

During tempeh production, the mold causes striking compositional changes in the soybean substrate. Some of the changes in soluble solids and nitrogen have been investigated (Van Veen and Schaefer, 1950; Boorsma, 1900) and are still under study (Steinkraus *et al.*, 1960), but little is known concerning changes in the lipids. Since soybeans are rich in lipid matter (20–26% of the dry weight) this class of compounds is worthy of consideration.

The present study was an outgrowth of the investigations of Steinkraus *et al.*, who were concerned with production of soybean tempeh under carefully controlled laboratory and pilot-plant conditions. Recent developments in the techniques of lipid fractionation and the use of vapor phase chromatography for separation of fatty acids have been applied to this study of the changes in composition of soybean lipids during the production of tempeh.

METHODS

Tempeh was produced from soybeans by the method of Steinkraus *et al.* Seneca soybeans, a medium-sized yellow variety, were soaked overnight in dilute lactic acid solution (100 g dry beans/3 ml of 85% lactic acid/300 ml water) at 25°C. The hydrated beans were drained and the soak water saved. The beans were run through a vegetable peeler (Reynolds Electric Co., Chicago) to loosen the skins. The beans were then separated from the skins by water flotation. The skins were discarded and the beans were placed in stainless-steel pans to a depth of 1–1½ in. The beans were covered with the dilute-acid soak water and heated 90 minutes at 100°C. The soak water was then drained from the beans and discarded. The beans were cooled to about 37°C, inoculated with spores of *R. oryzae* (1 g dried-bran culture per kg of cooked beans) and mixed to ensure inoculation of the entire bean mass. The inoculated beans were then incubated at 37°C.

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Samples of the fermenting soybeans were removed at 0, 20.5, 22, 24, 25, 26, 27, 28.25, 29.5, 30, 48, and 69 hours. The 20.5-hour sample represented the beginning of visible mold growth throughout the bean mass, which was accompanied by a rise in temperature above that of the incubator. During active mold growth, the pH of the samples was recorded as an estimation of the amount of mold activity that had occurred at the time the sample was taken. Each sample was frozen at −20°C and lyophilized before extraction of the lipids. The lyophilized tempeh samples were ground to a fine powder, and 10.0-g samples were extracted with diethyl ether for 24 hours in Soxhlet extractors. The ether was evaporated, the weight of total ether extract determined, and the concentration of free fatty acids (acid number) measured by titration with standard alkali.

Free fatty acids were separated by the method of Mattick and Lee (1959). Acid numbers were determined by the method of Lee and Wagenknecht (1951). Unsaponifiable material and neutral-fat fatty acids were determined by the method of Scholfield and Dutton (1955). Fatty acids were methylated with diazomethane according to Roper and Ma (1957). The diazomethane was prepared from nitrosomethylurea, which was prepared by the method of Amstutz and Myers (1943). Lipase activity was measured manometrically at 37°C (Martin and Peers, 1953). One-gram samples of lyophilized tempeh were ground with 9 ml of 0.0263*M* $NaHCO_3$ buffer in a glass mortar. One-milliliter samples of the resulting slurry were pipetted into the main compartment of Warburg flasks. The substrate (0.5 ml of 2% tributyrin w/v in 0.0148*M* $NaHCO_3$ buffer) was placed in the side-arm of the flasks. Total volume of reactants was 3.0 ml.

An Aerograph gas chromatographic instrument, Model A-100, was employed for fractionation, identification, and quantitative determination of the methyl esters of the fatty acids. A 5-ft column, packed with Craig polyester succinate (Craig and Murty, 1959), was maintained at 235°C, with helium used as the carrier gas at a flow rate of 50 ml per minute. The individual methyl esters were identified by comparing retention times with those of standards that consisted of a series of methyl esters of known fatty acids.

RESULTS

The concentration of total ether-extractable lipid rose slightly at the time of most active mold growth (20–30 hr) and then diminished. The concentration of titratable acids increased throughout fermentation (Table 1). The acid number increased from an initial value of 1.7 to 78.3 after 69 hours at 37°C. The pH of the fermenting soybeans showed a steady increase despite the relatively large amount of acid being liberated. This was presumably due to liberation of ammonia or other basic end products of protein decomposition.

Table 1. Time course of changes in lipids and of lipase action in soybeans during the production of tempeh.

Hours at 37°C	Total ether extract: % of dry weight	Total ether extract: Acid number	pH	Lipase Q_{CO_2} [a]
0	24.57	1.7	4.95	0
20.5	25.90	16.3	6.22	1.54
22	26.11	15.3	6.20	2.46
24	26.20	19.7	6.00	2.66
25	26.28	23.3	6.52	2.38
26	26.46	28.5	6.03	1.70
27	26.65	22.9	6.20	1.14
28.25	26.23	25.1	6.23	1.92
29.5	26.19	28.2	6.40	1.34
30	25.72	29.3	6.20	2.90
48	24.37	55.6	7.10	0.96
69	22.33	78.3	7.30	2.00

[a] $Q_{CO_2} = \mu l$ of CO_2 released per mg dry weight per hour.

Lipase activity was readily demonstrable at all stages of the tempeh fermentation when tributyrin was employed as the substrate. Because the soybeans had been heated, which inactivated the intrinsic enzymes, liberation of fatty acids during fermentation was caused by action of the lipase of *R. oryzae* (Table 1).

To identify the fatty acids liberated during fermentation, samples were taken at 24, 30, 48, and 69 hours for analysis. These samples were extracted for 48 hours with diethyl ether, the solvent removed, and the weight of total ether extract determined. A sample of lyophilized cooked beans, treated in the same manner, served as a control.

The total ether-extractable lipid was dissolved in a mixture of diethyl ether, petroleum ether, and 95% ethanol (70:70:20) and shaken three times with 35-ml portions of 1% Na_2CO_3 (Mattick and Lee, 1959). The combined Na_2CO_3 solutions containing the free fatty acids in the form of their sodium salts were then acidified with 2*N* HCl. The fatty acids were recovered by extraction with diethyl ether, the solvent was removed, and the acids dried to constant weight *in vacuo* over P_2O_5.

The fatty acid composition of the neutral fat in the cooked bean sample was determined in the following manner. Following the removal of free fatty acids by washing with 1% Na_2CO_3 solution as described above, the neutral-fat fraction was recovered by evaporation of the organic solvent mixture, and weighed. The fatty acids of the neu-

tral fat were liberated by saponification with aqueous KOH, followed by acidification of the mixture after removal of unsaponifiable material (Scholfield and Dutton, 1955). After removal of ether, the acids were dried to constant weight *in vacuo* over P_2O_5.

The free fatty acids and neutral-fat fatty acids obtained from tempeh were methylated with diazomethane, using the micro-apparatus of Roper and Ma (1957). A sample of the fatty acid to be methylated (40–60 mg) was placed in a 2-ml centrifuge tube, dissolved in about 1 ml of ether, and positioned under the diazomethane delivery capillary tube. Diazomethane was generated and bubbled through the tube containing the fatty acid sample.

The methylated fatty acids were allowed to stand at room temperature until the ether had evaporated. They were then dissolved in small amounts of ether (0.1–0.2 ml) for injection into a gas chromatography apparatus.

It has been suggested that diazomethane might be unsatisfactory for methylating unsaturated fatty acids because pyrazoline ring compounds might form by addition across double bonds (Stoffel *et al.*, 1959). A sample of oleic acid was methylated with diazomethane by the method described above. The sample was examined by gas chromatography. The major peak was found in the same place on the chromatogram as was authentic methyl oleate; minor peaks were assumed to be impurities in the oleic acid. These results showed that methyl oleate was formed by reaction of oleic acid and diazomethane. The chromatography of fatty acids from the inositol phosphatide fraction of snapbeans and from samples of insect lipids methylated in this laboratory by this procedure has shown normal peaks for the methyl esters of linoleic and linolenic acids. The diazomethane procedure was therefore considered suitable for methylating these acids.

The methyl esters of the fatty acids were identified by comparing their retention times with those of authentic compounds. Also used to identify the methyl esters were the ratios of the retention times as compared to an internal standard (methyl palmitate). The area under each peak is directly proportional to the molar concentration of each component. The area relationship can be used for calculating relative concentrations of components present as mol per cent or mol fraction.

Table 2 gives the concentrations of free fatty acids liberated by the lipase of *R. oryzae* during tempeh fermentation. About one-third of the total ether-extractable soybean lipid was hydrolyzed by the mold in 69 hr of incubation. The concentrations and types of fatty acids present in the free form in tempeh correspond to those of the neutral-fat fraction of the heated soybeans that were not fermented. A nearly twofold increase in palmitic acid was noted in the samples taken at 24 and 30 hr of incubation, but in the later stages of fermentation the concentration of palmitic acid decreased to the initial concentration. About 40% of linolenic acid was utilized by the *R. oryzae*, but there was no apparent preferential utilization of any other fatty acid (Table 2).

DISCUSSION

The action of the tempeh mold may be considered to be primarily that of rendering the various components of the soybean more digestible as a result of enzyzmic digestion of proteins, carbohydrates, and fats (Van Veen and Schaefer, 1950). Steinkraus *et al.* (1960) noted that the soluble solids rose from 13 to 21% during the most rapid mold growth, and continued to rise, to 27.5% at the end of the fermentation. Soluble nitrogen rose from 0.5 to nearly 2%, whereas total nitrogen remained relatively constant (about 7.5%). Reducing substances showed

Table 2. Distribution of free fatty acids during soybean tempeh fermentation.

Sample	Mol per cent					Mg/100 g of tempeh						
	Palmitic	Stearic	Oleic	Linoleic	Linolenic	Palmitic	Stearic	Oleic	Linoleic	Linolenic	Total g/100 g of tempeh	% of total ether extract
Cooked soybean, neutral fat	10.86	3.90	18.11	61.00	6.13							
Cooked soybean, free fatty acids	22.24	15.21	62.53	0	0	41	31	127	0	0	0.26	1.09
24-hour tempeh	19.81	3.75	15.39	54.62	6.43	420	175	713	2510	293	3.59	13.87
30-hour tempeh	18.23	4.31	17.24	55.08	4.45	771	202	802	2543	204	4.77	18.93
48-hour tempeh	10.83	2.96	20.07	61.57	4.56	665	202	1359	4138	304	6.93	30.00
69-hour tempeh	11.37	4.36	19.98	60.62	3.67	863	367	1671	5032	302	8.19	35.11

a decline, being utilized by the mold in its metabolism, and did not regain their initial level even though enzymic breakdown of higher carbohydrates continued throughout the incubation period.

Boorsma (1900) reported a marked decrease in fat content during the fermentation, and noted that the extracted fat was quite solid. This observation led to the supposition that the fungus acted primarily on the unsaturated fatty acids. Van Veen and Schaefer (1950) examined the total ether extracts of tempeh from Javanese and Manchu varieties of soybeans, and found lower total fat levels than those of the corresponding cooked beans. They further observed that the extracted fat contained numerous small solid particles, whereas the extracted fat of raw beans was a clear oily liquid. In the present study the total content of ether-extractable fat remained fairly constant throughout the tempeh fermentation, ranging from 22.3 to 26.7% of the dry weight. The relative ratios of fatty acids changed as fermentation proceeded, yet *R. oryzae* failed to degrade the liberated fatty acids.

The free fatty acids of the cooked soybeans were present in such small amounts that their presence was not considered of significance in any consideration of the changes brought about in the lipids of soybeans during the production of tempeh. There was no evidence for the presence of linoleic or linolenic acids, even in trace amounts, and the quantities of the three acids present were considerably different from those in either cooked-bean neutral fat or the various samples of tempeh.

Roughly one-third of the neutral fat of the soybean was hydrolyzed by the fungal lipase during the production of tempeh. Linoleic acid was the major component of the free fatty acids, just as it is in the unhydrolyzed neutral fat of the cooked soybeans. The fact that very little if any of the linoleic acid was lost through utilization by the mold may be of nutritional significance, since this is one of the essential fatty acids.

Although fatty acids were liberated by the hydrolysis of soybean lipid, there was no subsequent utilization of these fatty acids. It can be concluded, therefore, that *R. oryzae* either does not possess the enzyme systems necessary for metabolizing these fatty acids or that *R. oryzae* is impermeable to these acids under the conditions of tempeh fermentation.

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Dependency of Almond Preference on Consumer Category and Type of Experiment [a]

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SUMMARY

Three almond selections with distinct tastes and other characteristics were compared by male and female tasters in different types of experiments. The tasters differed in preferences and in distributions of recorded degrees of liking. Interactions between sex, almond selection, and type of experiment were present and important.

INTRODUCTION

Consumer preferences between kernels of three selections of almonds were studied in the spring of 1960. Used as representative consumers were students and staff of the University of California at Davis. The study aim was threefold: 1) to determine if there was distinct preference for selection of almond; 2) to compare reactions to almonds tasted in paired situations with reactions to almonds tasted in isolated (single) situations; 3) to determine the reliability of single-test consumer studies by analysis of the data by days, time of day, and sex of tasters.

The results were unexpected, and have important implications for consumer preference testing.

THE EXPERIMENT

The kernels were obtained from the 1959 crop at the University's Wolfskill Experimental Orchard, near Winters, California. The almonds were unnamed selections developed in the University's almond breeding project. Preliminary testing had assessed all three selections as "acceptable." Their tastes, however, were distinctly different, as were the characteristics of their kernels. Figure 1 shows the general shape, appearance, and size of the kernels.

Kernels of *selection 25-26* were medium-sized, flat, and broadly rounded. The color was medium brown, with a dull appearance. The pellicle bore numerous short fine hairs (pubescence) that contributed to the dullness. Also present was a network of slightly darker veins. Kernels of *selection 6A-11* were somewhat larger than the others, relatively flat, and somewhat elongated. The color was golden-brown, with prominent dark-brown veins interspersed throughout the pellicle. The kernel had a bright appearance and was free of pubescence. Kernels of *selection 16-40* were the smallest, relatively long and narrow, but plump for their size. The color varied from light golden-brown to dark-brown, and the veins were inconspicuous. The pellicle was smooth, without pubescence.

Almonds of a single selection were placed in glassine bags, 3 per bag, and coded for paired or single-sample experiments. All samples for a given consumer were placed in a larger bag, along with coded score sheets (Table 1). Each taster was given enough bags and score cards for a five-day twice-a-day test (4 times a day in the single-sample test). The test bags were distributed to students in two fraternities and two residence halls, and to staff members of three departments. Care

Fig. 1. Kernels of the three almond selections in the test. Left, selection 25-26; center, selection 6A-11; right, selection 16-40. Bottom row, flat side; top row, narrow side view.

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[a] Joint contribution of the Departments of Mathematics, Viticulture and Enology, and Pomology, University of California, Davis.

Table 1. Score cards for consumer survey on almonds.

PAIRED SAMPLES

This is a test on flavor preference for two kinds of almonds. We would like you to taste the contents of the two bags on the dates and times indicated below. *It is important that you taste all ten sets in the order given and over a period of 5 days.* Please record the actual time of tasting in the right-hand margin.

Suppose the two bags were numbered 29 and 67. Taste the almonds from each bag and circle the number whose contents you prefer and also circle the degree of liking of each. Thus, if you preferred 67 over 29 and liked 67 very much and 29 slightly:

	Bag Nos.	Degree of liking	Actual time tasted	
Example:				
May x	29	dislike, slight, much	May x	7 PM
I prefer	or			
	67	dislike, slight, much		
May 16 AM	42	dislike, slight, much	______	
I prefer	79	dislike, slight, much	______	
May 16 PM	58	dislike, slight, much	______	
I prefer	26	dislike, slight, much	______	

SINGLE SAMPLES

This is a test on flavor preference for two kinds of almonds. We would like you to taste one type of almond at a time four times per day according to the schedule below. The bags are numbered and labeled as to date and time. Record the actual time of tasting at the right and circle the degree of liking. *It is important that each bag be tested on a separate occasion and in no case should two bags be tasted within one hour of each other.*

Suppose the bag sample was numbered 23 and you liked it slightly:

Date and time	Bag No.	Degree of liking	Actual time tasted	
Example:				
May x AM	23	dislike, slight, much	May x	8 AM
May 16 early AM	41	dislike, slight, much	______	
late AM	62	dislike, slight, much	______	
early PM	55	dislike, slight, much	______	
late PM	96	dislike, slight, much	______	

was taken to give each group its proper proportion of single-test or paired-test samples.

The score sheets for the paired experiment called for preference between the two samples, and a rating of dislike, like slightly, or like much. Each taster recorded date and time of tasting (morning and afternoon). Before the test, each participant was given oral instructions on procedure and use of the score cards. Actual time of tasting was recorded so as to prevent large deviations from the daily schedule. Score sheets and instructions were similar for the single-sample experiment (Table 1). In the single-sample test the subjects were urged to space both the morning and afternoon tastings widely.

Of the samples distributed, complete returns were made by 125 (52%). This response is considered reasonable where students are not forced to give answers. Forcing answers may lead to responses not related to actual preference.

Selection 25-26 was compared to *6.4-11* and to *16-40* in paired and single sample situations. The complete experiment can be thought of as being composed of 8 independent sub-experiments if the sexes are considered separately.

Experimental Data and Their Analysis

Tables 2 and 3 present results of the paired preference and rating experiments; the single-sample results are in Tables 4 and 5. Only the data sheets that were complete are considered; incomplete sheets (also listed and summarized) did not noticeably affect the general picture.

The two considerations on preference in Tables 2 and 3 that are of interest are: is there a preference, and, do the tasters have the same preferences?

For males of Table 2, adjusted chi-square (Yates') for preference is 4.41 with one degree of freedom, which is significant at the 5% level. The interaction chi-square calculated for the hypothesis 4.0 and 6.0 expected is 14.19 with 11 degrees of freedom, which is definitely not significant. Hence, the males preferred *6.4-11* over *25-26*.

For the females of Table 2, the chi-square for preference is practically zero. Testing the individual tasters against the hypothesis of equal preference, we find chi-square equal to 18.80 with 10 degrees of freedom, which has a probability value a little less than 5%. Two tasters show unusual preference for *25-26*, and two show somewhat unusual preference for *6.4-11*. A point of prime importance in all general consumer acceptance testing, as opposed to very artificial laboratory testing, is that the direction of preference is not specific and that many persons are indifferent to the tested characteristic. These facts are discussed more fully by Baker *et al.* (1960).

For the males of Table 3, adjusted chi-square for preference is 1.70 with one degree of freedom, which is insignificant. The interaction chi-square for tasters if we use 5 and 5 as expected values is 32.40 with 16 degrees of freedom, which is significant at the 1% level. Four males express unusual preference for *25-26* and one for *16-40*.

For the females of Table 3, adjusted chi-square for preference is 6.40 with one degree of freedom, which is significant at the 2% level. Thus, in testing for taster-by-selection interaction, we use 3.9 and 6.1 as expected values. The interaction chi-square is 28.96 with 14 degrees of freedom, which is significant at the 2% level. Hence, on the whole, the females prefer *16-40*, but there is one violent

Table 2. Preference and degree of liking of almond selections 25-26 and 6A-11 (paired experiment).

	Preference		Degree of liking							
			25-26				6A-11			
Taster	25-26	6A-11	D [a]	S	M	C	D	S	M	C
			Male							
7	7	3	2	3	5	21	3	7	0	14
9	5	5	1	9	0	18	2	8	0	16
11	5	5	0	7	3	23	1	5	4	22
13	4	6	0	7	3	23	0	5	5	25
19	3	7	1	7	2	20	1	3	6	24
20	3	7	4	5	1	13	1	5	4	22
23	3	7	0	7	3	23	0	3	7	27
41	3	7	2	6	2	18	1	5	4	22
45	4	6	2	6	2	18	2	7	1	17
47	1	9	5	4	1	11	2	2	6	22
55	3	7	5	3	2	12	2	5	3	19
58	7	3	0	10	0	20	1	9	0	18
Total 12	48	72	22	74	24	220	16	64	40	248
			Female							
3	4	6	0	9	1	21	0	7	3	23
27	9	1	0	4	6	26	3	6	1	15
29	6	4	0	4	6	26	0	6	4	24
30	2	8	5	4	1	11	0	6	4	24
32	6	4	1	5	4	22	2	4	4	20
33	5	5	0	6	4	24	1	5	4	22
34	2	8	2	6	2	18	0	4	6	26
37	5	5	3	2	5	19	2	4	4	20
39	4	6	0	6	4	24	0	4	6	26
59	5	5	0	10	0	20	0	6	4	24
60	8	2	2	6	2	18	6	3	1	9
Total 11	56	54	13	62	35	229	14	55	41	233

[a] D, dislike; S, like slightly; M, like much; and C, combined score $(0 \times D + 2 \times S + 3 \times M)$.

exception (taster 142) and a less severe one (taster 155).

For the single-sample experiments (Tables 4 and 5) chi-squares for differences between almonds are insignificant, but the difference between male and female preferences is significant (chi-square is 9.96 with 2 degrees of freedom, and hence $P<1\%$).

The scoring scale of dislike, like slightly, and like much, obviously omits the zero point (neither like or dislike). Thus the scale should perhaps go $-1, 1, 2$; or (preferred for our purposes) $0, 2, 3$—a shift of one unit to the right. For a fuller discussion of the problem of scaling see Scheffé (1952). Thus the combined liking scores of Tables 2, 3, 4, and 5 are computed as $0 \times D + 2 \times S + 3 \times M$, in which D is the number of dislike ratings, S is the number of like slightly, and M is the number of like much.

Table 6 gives the ordinary Pearsonian product-moment correlation coefficients by sex for the data of the four tables. These 8 experiments are independent of each other. For tests of significance we can transform the r's to Z's (Alder and Roessler, 1960), and then we have approximately normally distributed variables with known standard deviations. On this basis it is apparent that the single and paired situations are essentially different. Further, the correlation coefficients are higher for the males than for the females ($t = 2.539$, $P<.01$, two-tailed test).

The single and joint distributions of the values of the combined liking scores (C) are not markedly non-normal so there is no reason to question the applicability of the usual correlation coefficients, t-test, and F-test.

For almost all categories there are pronounced interactions between taster and degree of liking (Table 7). These interactions were calculated from data in Tables 2, 3, 4, and 5. These interactions may be less for females than for males. The chi-

squares can be compared by the F-test, and the ratios of the corresponding chi-squares are suggestive of a sex difference. The smaller interactions for females (Table 7) are not in conflict with the smaller correlation coefficients for females (Table 6), because less interaction means more over-all conformity, and hence less correlation between combined scores, as can be verified by the scatter diagrams of the C-scores. The interactions between tasters and degree of liking mean that the tasters vary a great deal in recorded ratings of degree of liking for almonds. The observed interactions are not fully explained, however, by the simple statement that some tasters like almonds and some do not. The additional consideration seems to be, in any paired comparison (e.g., A vs. B), that the tasters can be divided into three categories: those who prefer A, those who prefer B, and those with no preference.

Table 8 gives the means and variances of the combined scores in Tables 2, 3, 4, and 5. Several

Table 3. Preference and degree of liking of almond selections 25-26 and 16-40 (paired experiment).

	Preference		Degree of liking							
			25-26				16-40			
Taster	25-26	16-40	D [a]	S	M	C	D	S	M	C
			Male							
123	2	8	0	8	2	22	0	2	8	28
132	6	4	0	5	5	25	1	5	4	22
133	4	6	0	6	4	24	0	5	5	25
135	3	7	0	7	3	23	0	2	8	28
140	7	3	1	2	7	25	1	7	2	20
147	5	5	0	5	5	25	1	4	5	23
149	4	6	1	7	2	20	0	6	4	24
161	6	4	2	4	4	20	2	6	2	18
162	8	2	0	3	7	27	2	4	4	20
163	7	3	0	6	4	24	3	4	3	17
164	3	7	6	4	0	8	5	4	1	11
165	4	6	4	2	4	20	2	6	2	18
166	8	2	0	2	8	28	2	6	2	18
170	4	6	0	4	6	26	1	1	8	26
171	5	5	1	7	2	20	0	6	4	24
173	9	1	0	4	6	26	0	8	2	22
177	9	1	0	5	5	25	3	5	2	16
Total 17	94	76	15	81	74	388	23	81	66	360
			Female							
121	5	5	4	5	1	13	2	6	2	18
122	1	9	5	5	0	10	1	7	2	20
124	6	4	0	8	2	22	3	3	4	18
129	5	5	1	6	3	21	2	3	5	21
130	4	6	2	8	0	16	0	10	0	20
142	8	2	2	4	4	20	3	7	0	14
144	5	5	1	4	5	23	1	2	7	25
151	2	8	4	4	2	14	1	5	4	22
152	3	7	2	7	1	17	2	4	4	20
153	1	9	2	5	3	19	1	4	5	23
154	5	5	2	5	3	19	0	6	4	24
155	7	3	1	4	5	23	1	7	2	20
156	4	6	0	6	4	24	0	3	7	27
159	1	9	4	5	1	13	1	3	6	24
180	2	8	4	5	1	13	2	6	2	18
Total 15	59	91	34	81	35	267	20	76	54	314

[a] D, dislike; S, like slightly; M, like much; and C, combined score $(0 \times D + 2 \times S + 3 \times M)$.

Table 4. Degree of liking of almond selections 25-26 and 6A-11 (single sample).

Taster	Degree of liking							
	25-26				6A-11			
	D	S	M	C	D	S	M	C
				Male				
61	0	10	0	20	0	8	2	22
62	3	6	1	15	4	3	3	15
63	2	7	1	17	2	8	0	16
64	0	5	5	25	0	6	4	24
69	0	7	3	23	0	3	7	27
71	1	6	3	21	0	10	0	20
73	1	6	3	21	3	3	4	18
75	2	5	3	19	5	4	1	11
76	0	5	5	25	0	6	4	24
78	3	5	2	16	2	5	3	19
79	1	7	2	20	1	7	2	20
80	3	3	4	18	4	4	2	14
85	0	2	8	28	0	4	6	26
88	0	1	9	29	0	9	1	21
102	5	4	1	11	4	5	1	13
103	4	3	3	15	5	4	1	11
105	2	6	2	18	1	7	2	20
108	2	3	5	21	1	3	6	24
109	0	4	6	26	0	6	4	24
110	4	3	3	15	3	5	2	16
111	2	6	2	18	2	4	4	20
112	1	7	2	20	2	3	5	21
113	2	6	2	18	0	8	2	22
116	0	3	7	27	0	8	2	22
119	3	7	0	14	1	8	1	19
120	0	5	5	25	0	6	4	24
Total 26	41	132	87	525	40	147	73	513
				Female				
65	0	9	1	21	0	5	5	25
70	0	10	0	20	0	9	1	21
81	0	4	6	26	0	2	8	28
83	1	7	2	20	1	6	3	21
89	0	2	8	28	0	6	4	24
92	3	4	3	17	4	4	2	14
93	1	7	2	20	3	3	4	18
94	1	4	5	23	1	7	2	20
95	3	5	2	16	1	6	3	21
98	0	3	7	27	2	6	2	18
99	0	3	7	27	0	4	6	26
114	1	2	7	25	0	2	8	28
115	0	1	9	29	0	1	9	29
Total 13	10	61	59	299	12	61	57	293

Table 5. Degree of liking of almond selections 25-26 and 16-40 (single sample).

	Degree of liking							
	25-26				16-40			
Taster	D	S	M	C	D	S	M	C
				Male				
188	2	8	0	16	1	7	2	20
194	1	4	5	23	2	4	4	20
195	0	0	10	30	0	0	10	30
196	0	2	8	28	0	7	3	23
200	0	6	4	24	0	4	6	26
203	1	4	5	23	1	6	3	21
204	3	6	1	15	3	6	1	15
206	0	4	6	26	1	4	5	23
223	4	5	1	13	2	6	2	18
225	3	4	3	17	2	6	2	18
226	4	2	4	16	4	5	1	13
230	0	5	5	25	0	5	5	25
238	2	8	0	16	2	3	5	21
240	2	5	3	19	1	8	1	19
Total 14	22	63	55	291	19	71	50	292
				Female				
201	5	5	0	10	3	5	2	16
205	0	2	8	28	0	4	6	26
207	1	6	3	21	0	5	5	25
208	2	5	3	19	2	4	4	20
209	0	7	3	23	3	5	2	16
211	0	5	5	25	0	3	7	27
212	1	6	3	21	2	5	3	19
213	2	4	4	20	3	2	5	19
214	1	8	1	19	1	9	0	18
216	2	5	3	19	1	4	5	23
217	0	10	0	20	3	3	4	18
218	0	4	6	26	0	3	7	27
220	1	7	2	20	1	2	7	25
231	3	4	3	17	1	4	5	23
233	0	4	6	26	3	2	5	19
234	1	3	6	24	3	6	1	15
235	3	5	2	16	0	7	3	23
Total 17	22	90	58	354	26	73	71	359

significant comparisons can be made. For instance, for the males the combined score mean for *25-26* (paired with *6.4-11*) is significantly different from the mean for *25-26* (paired with *16-40*). For the females the mean for *25-26* (paired with *16-40*) is different from the mean for *25-26* (single experiment but tasted in conjunction with *6.4-11*). These and other obvious comparisons indicated complicated interactions between the kind of almonds, type of experiment, and sex of taster.

In considering Table 8 we must bear in mind that we are dealing with 8 independent sub-experiments and that the means of combined scores really measure consumer acceptability of different selections under distinctly different tasting situations. On that basis, we are justified in noting significant differences indicating that some situations are different from others. In testing for significance the usual t-test can be used in testing for differences between independent situations. In cases of considerable correlation between the combined scores for two selections tasted by the same individuals, the test for difference would be made more sensitive by allowing for the positive correlation.

Table 9 assesses the effectiveness of these experiments in detecting liking differences between almond selections. All of the comparisons appear of doubtful significance except when the females

compared *25-26* with *16-40*. In that case, the chi-square is 7.85 with *2* degrees of freedom, and hence P<*2%*. The kernels of *16-40* have a different shape and are smooth. It is emphasized again that taste differed distinctly among these almond selections.

Calculation of chi-squares for the data indicate that day of tasting and time of day produced no detectable differences.

DISCUSSION

The findings support the view developed in previous publications (Baker *et al.*, 1954, 1958, 1960; Mrak *et al.*, 1959) that the wide variation in response of individuals must be fully considered in sensory tests. They further imply that there is no such thing as preference independent of qualifying conditions. Preference must be considered as dependent not only on the particular character examined but also on every circumstance surrounding and involved in the evaluation.

Table 6. Correlation coefficients for combined scores for paired and single-sample experiments.

Selections	Single		Paired	
	Male	Female	Male	Female
25-26 and 6A-11	0.717	0.633	0.193	−0.034
25-26 and 16-40	0.817	0.333	0.407	0.250

A great deal of so-called consumer preference testing is conducted in the laboratory under rigidly controlled conditions with trained observers who usually prefer the same differences. Such testing does not have much connection with real-life situations of going into a retail store and selecting something to consume. The present study emphasizes the difference in direction of preference of consumers, and the large proportion of consumers that are indifferent to the test situation. From this point of view it is interesting and important to note the distinct effect of sex on preference. This preference seems to be based on a shape difference that has little to do with taste. Such a finding has not been reported before, as far as we know.

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Table 7. Interaction chi-squares for tasters and degrees of liking by sex, selection of almond, and type of experiment.

Category	Chi-square	Degrees of freedom	Probability value
Male			
1 25-26 paired	41.204	22	<.01
2 6A-11 paired	37.117	22	<.02
3 25-26 paired	79.469	32	<.01
4 16-40 paired	56.012	32	<.01
5 25-26 single	89.625	50	<.001
6 6A-11 single	100.746	50	<.001
7 25-26 single	60.960	26	<.01
8 16-40 single	47.013	26	<.01
Female			
1 25-26 paired	46.054	20	<.01
2 6A-11 paired	38.114	20	<.01
3 25-26 paired	37.053	28	<.10
4 16-40 paired	42.178	28	<.05
5 25-26 single	62.456	24	<.001
6 6A-11 single	56.118	24	<.001
7 25-26 single	61.052	32	<.01
8 16-40 single	47.345	32	<.05

Table 8. Means and variances of the combined scores[a] by sex, selection of almond, and type of experiment.

Category	Number	Mean	Variance
Male			
1 25-26 paired	12	18.333	18.242
2 6A-11 paired	12	20.667	15.152
3 25-26 paired	17	22.824	21.154
4 16-40 paired	17	21.176	20.779
5 25-26 single	26	20.192	22.001
6 6A-11 single	26	19.731	19.485
7 25-26 single	14	20.786	29.412
8 16-40 single	14	20.857	19.516
Female			
1 25-26 paired	11	20.818	19.164
2 6A-11 paired	11	21.182	26.364
3 25-26 paired	15	17.800	19.743
4 16-40 paired	15	20.933	11.067
5 25-26 single	13	23.000	18.500
6 6A-11 single	13	22.538	20.769
7 25-26 single	17	20.824	19.029
8 16-40 single	17	21.118	16.110

[a] $(0 \times D + 2 \times S + 3 \times M)$.

Table 9. Differences in preferences among almond selections, by sex and type of experiment.

		25-26			6A-11		
		D	S	M	D		M
Paired:	Male	22	74	24	16	64	40
	Female	13	62	35	14	55	41
Single:	Male	41	132	87	40	147	73
	Female	10	61	59	12	61	57
	Total	86	329	205	82	327	211
		25-26			16-40		
		D	S	M	D	S	M
Paired:	Male	15	81	74	23	81	66
	Female	34	81	35	20	76	54
Single:	Male	22	63	55	19	71	50
	Female	22	90	58	26	73	71
	Total	93	315	222	88	301	241

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Anthocyanin Pigment in Passion Fruit Rind

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SUMMARY

Detailed studies were reported on isolation and identification of the pigments naturally occurring in passion fruit rind (*Passiflora edulis* Sim.) From color tests, distribution number, paper chromatography, sugar residue, and absorption spectra, pelargonidin 3-diglucoside reported in passion fruit rind is about 1.4 mg of the pigment per 100 g of the fresh material.

Earlier reports (Pruthi, 1955; Pruthi and Lal, 1955) indicated that purple passion fruit (*Passiflora edulis* Sims) has a very short storage life (7–10 days) at room temperature, or 4 weeks at 42–45°F, during which period the outer purple pigment in the rind undergoes degradation. It was felt that the characteristic purple of the rind may be due to the presence of an anthocyanin pigment and that the color changes during storage may be attributed mostly to degradation of the pigment itself. Full understanding of the exact nature of the changes taking place during storage requires knowledge of the chemistry of the pigment and the factors responsible for its degradation. A survey of the literature (Pruthi, 1955) revealed little information on the nature of the pigments in the rind, though the pigments in passion fruit juice were recently reported on (Pruthi and Lal, 1958).

Qualitative tests revealed traces of chlorophyll, carotenoids, xanthophylls, flavones, and leuco-anthocyanins in passion fruit rind, and anthocyanins in considerable amount. Detailed studies were undertaken on isolation, purification, and characterization of the anthocyanins occurring naturally in passion fruit rind. The results are presented here.

EXPERIMENTAL

Isolation of the pigment. Frozen passion fruit rind (minced) in 500-g lots was thawed and macerated with 1% methanolic HCl (1 L) in a Waring blender. The solution was allowed to stand for about ½ hr and filtered under low pressure. The residue was re-extracted with further portions of methanolic HCl. Extraction of the pigments, after presoaking in 1% methanolic hydrochloric acid as followed by Brown (1940) was also tried. It was found, however, that soaking ½ hr and extracting the pigment 3–4 times removed most of the anthocyanins. The combined extracts were concentrated under vacuum to about ¼ volume. A saturated solution of neutral lead acetate was then added to it in excess until no further precipitation took place. The heavy precipitate was removed by centrifuging 10 min at 2500 rpm. The precipitate was washed with ethyl alcohol and then suspended with vigorous stirring in methanol, and conc. HCl was added until the entire precipitate had decomposed. The white lead chloride precipitate was filtered off. The red anthocyanin solution was concentrated under vacuum until a precipitate began to form. It was then added with vigorous stirring to three times its volume of anhydrous ether. The sludge was removed by filtration, redissolved in methanol, and added to a further quantity of anhydrous ether. After precipitating the anthocyanin 3–4 times, it was dried under vacuum at 45°C, and the crude pigment thus obtained was used for chromatographic studies.

Purification of the pigment. The crude pigment obtained was further purified by the picrate method as adopted by Sondheimer and Kertesz (1948) for purification of pelargonidin 3-glucoside. The noncrystalline chloride obtained (yield about 21%) was then crystallized with Willstatter and Burdick's method for the crystallization of callestephin chloride.

Identification of the pigment. *Preliminary identification.* 1) *Color tests.* In our present studies, the color tests evolved by Robertson *et al.* (1928) and certain specific tests suggested by Sondheimer and Kertesz (1948) for strawberry anthocyanins were carried out. The anthocyanidin obtained after hydrolysis of anthocyanins, and also the eluted aglycones from paper chromatograms were used for studying color reactions. Color tests revealed that

the pigment in passion fruit skin may be pelargonidin.

2) *Distribution number.* The color tests are of some value in characterizing the anthocyanins, but the distribution number (Perkin and Everest, 1915) is quite valuable in determining the degree and to some extent the type of glycosidation present in the pigment.

In this case, the distribution ratio of the pigment in amyl alcohol and aqueous phase was found to be 1:2, which indicated the possibility of a diglucoside of pelargonidin in passion fruit rind.

Identification by paper chromatography. 1) *Anthocyanins.* Although qualitative tests give an indication of the pigment present, they have their limitations. So it becomes necessary to confirm the results by paper chromatography, absorption spectra, etc. This was studied as follows:

The crude pigment obtained by precipitating with ether was used in the present studies. Whatman No. 1 filter paper was employed, and the following solvents systems were tried:

a) BAW = *n*-butanol–acetic acid–water, 4:1:5 (top layer) (Bate-Smith, 1949)

b) (Bu-HCl) = n-butanol–2N HCl, 1:1 (top layer) (Harborne, 1958 b)

c) Phenol, saturated with water (top layer) (Ponniah and Seshadri, 1953)

BAW was allowed to stand for three days before use, as recommended by Bate-Smith (1949). With all other solvents, fresh mixtures were used.

Using ascending, descending, and horizontal techniques, a number of chromatograms were run that gave one spot only, the R_f values of which corresponded well with those reported for pelargonidin (Table 1).

2) *Aglycones.* About 0.1 g of the crude material was refluxed for 5 min with 20 ml of 5N HCl. The solution was cooled and extracted twice with amyl alcohol. The aqueous layer, which was practically colorless, was neutralized with sodium bicarbonate. After concentrating, it was used to establish the identity of the carbohydrate or sugar residue.

The amyl alcohol extracts were combined, and a very small quantity was spotted on Whatman No. 1 filter paper. Before developing, the paper was saturated for 24 hr in chambers saturated with the solvent. The papers were developed by various solvents by the ascending and circular techniques. The following solvents were tried:

a) BAW (top layer)

b) Phenol saturated with water

c) "Forestal" solvent: acetic acid–conc. HCl–water (Bate-Smith, 1954) (30:3:10 v/v)-miscible.

Table 1. R_f values of the pigment in passion fruit rind in relation to those reported for pelargonidin in different solvent systems.

Paper-chromatographic techniques	Solvent system		
	BAW [a]	Bu-HCl [b]	Phenol saturated with water
For pigment in passion fruit rind			
Descending	0.59	......	......
Ascending	0.35	0.23	
Circular	0.50		0.75
Reported for pelargonidin			
Descending	0.59 [c]	0.26 [d]	0.73 [e]
Ascending	0.36 [d]	......	......
Circular	0.50 [e]		

[a] *n*-butanol–acetic acid–water.
[b] *n*-butanol–2N HCl.
[c] Bate-Smith, 1949.
[d] Harborne, 1958 a.
[e] Ponniah and Seshadri, 1953.

Of the three, the Forestal proved successful. The others were unsatisfactory because of tailing of the spots.

The R_f values of the aglycones in passion fruit rind in different solvent systems were quite comparable with those reported for pelargonidin in various plants. The R_f values of this pigment in three solvent systems compared well with those reported for pelargonidin glycoside (Bate-Smith, 1949, 1954; Ponniah and Seshadri, 1953).

3) *Sugar residue.* The carbohydrate residue obtained after hydrolysis of aglycone was neutralized with sodium bicarbonate and concentrated *in vacuo.* Aliquots of this solution were used for identifying the carbohydrate residue. Using an *n*-butanol–acetic acid–water system, the descending tecnique was employed. The chromatogram was developed for 3-4 days. The sugar residue was identified by spraying with benzidine trichloroacetic acid.

It was found that the carbohydrate resolved into a single spot corresponding to glucose. Since glucose and galactose have the same R_f values, it was considered essential to establish the identity of the sugar residue. This was done as follows: The

Table 2. R_f values of anthocyanidin (pelargonidin).

Solvent systems	R_f values	
	Ours	Others [a]
n-butanol–acetic acid–water	1.00	1.00
Phenol saturated with water	0.88	0.91
Forestal	0.72	......

[a] Ponniah and Seshadri, 1953.

chromatograms were developed along with two standard sugar solutions by the descending technique, and the solvent was allowed to drip from the paper (Jermyn and Isherwood, 1949) for a few days and then developed. In this case, there was a difference in the R_f values between the two sugars, thus simplifying identification of the sugar. Further, a specific spot test (Feigel, 1947) for glucose was performed to confirm the findings. Since the R_f values correspond with the reported values for pelargonidin 3-diglucoside, it may be concluded that it is a 3-diglucoside of pelargonidin.

Absorption spectra. The modified method of purification of the anthocyanin pigments by paper chromatography and their identification by spectral methods have recently been illustrated (Harborne, 1958 a). The same method was followed for the purification and identification of the pigment.

The pigment was extracted in methanolic HCl (97:3 v/v), centrifuged, and concentrated to a small volume. This extract was used for chromatographic purification of the pigment. The absorption spectra of the crude pigment were also studied. To study the development of the pigment during maturation of the fruit, the absorption spectra were determined of the methanolic HCl extracts of the pigment from

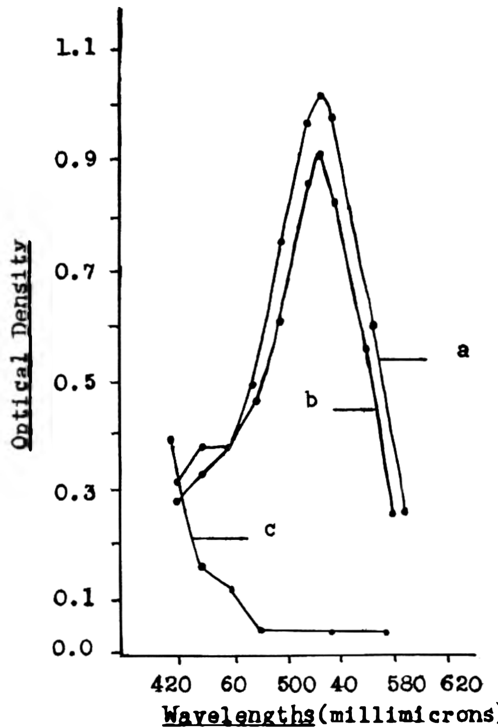

Fig. 1. The absorption spectra of the anthocyanin pigment from passion fruit rind at three stages of maturity: a) fully ripe; b) partially ripe; c) unripe (green).

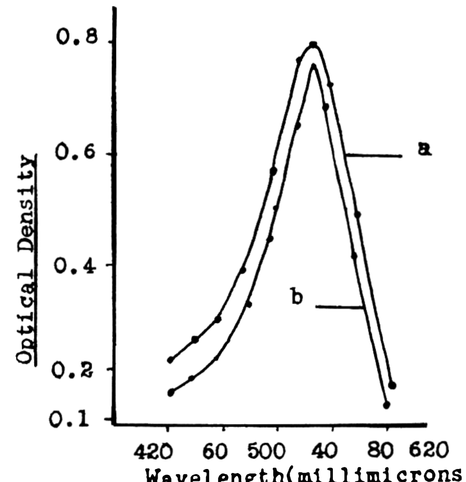

Fig. 2. The absorption spectra of the over-all and purified anthocyanin pigment from passion fruit rind: a) over-all pigment; b) purified pigment.

fruits at three different stages of maturity (green, partially ripe, and fully ripe). The results are in Fig. 1.

Whatman filter paper No. 1 was equilibrated for 24 hr before developing the chromatogram. The ascending technique was used. The spots were developed with *n*-butanol-acetic acid-water and *n*-butanol-2*N* HCl (1:1 v/v) for 48 hr. The chromatograms were dried, the bands were cut and eluted with water–methanol–acetic acid (25:70:5 v/v) (Harborne, 1958 a). The eluates were concentrated *in vacuo* and respotted, and the spots were developed with the same solvents. This process was repeated 3–4 times. Some of the final spots obtained were eluted with 0.01% methanolic hydrochloric acid, and the rest with 0.01% ethanolic HCl.

The anthocyanin under investigation had an absorption peak at 520 mμ in methanolic HCl solvent, and at 530 mμ in ethanolic HCl. These results compare well with those of Harborne (1958 a) for pelargonidin. Fig. 2 presents the absorption curve for the purified pigment, along with the absorption spectra for the over-all pigment. Fig. 3 presents the absorption spectrum of the purified pigment in the ultraviolet range. The purified pigment exhibited a peak at 270 mμ, and only a very weak absorption at 310 mμ, whereas the acylated compounds exhibited more than one distinct peak. From the above, it may be concluded that the passion fruit anthocyanin may not contain any acyl group.

1) *Quantitative estimation of the anthocyanin.* The measure of the intensity of light at the absorption maximum of the pigment (on the assumption that absorption follows Beer's law) is the principle

employed in quantitative estimation of anthocyanins. The method used was that developed by Sondheimer and Kertesz (1948) for quantitative measurement of anthocyanin concentrations in strawberry. The results are expressed as "Congo Red equivalent units" in mg%.

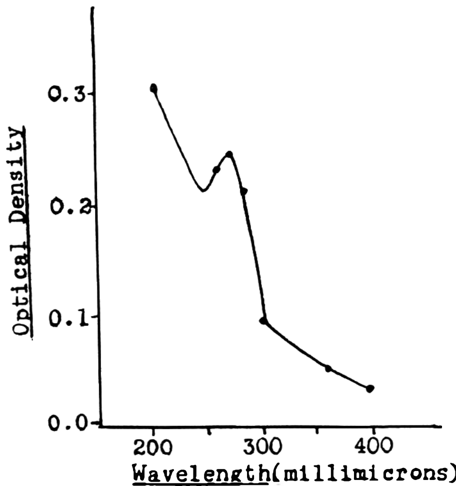

Fig. 3. Ultraviolet absorption curve for the purified anthocyanin pigment from passion fruit rind.

The results showed that passion fruit rind contained 1.4 mg of the pigment per 100 g of the fresh skin (or 9.33 mg/100 g on dryweight basis).

Acknowledgment

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Effects of Ionizing Radiations on Plant Tissues. V. Some Effects of Gamma Radiation on Lettuce Leaves[a]

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SUMMARY

The effect of gamma radiation from a Cobalt[60] source upon lettuce tissue was studied. The radiation-induced softening of leaf discs was assessed with an instrument designed to measure the bending of the disc under application of a given weight. It is estimated that the "threshold dose" of radiation-induced softening of lettuce is between 600 and 1,000 Kilorad. Lettuce leaf tissue thus appears more resistant to the softening effect of gamma radiation than the storage-type tissues of apples, carrots, or beets. The radiation-induced changes in the respiratory activity of lettuce were also studied, both during and after irradiation. Although both oxygen consumption and carbon dioxide evolution are stimulated during irradiation, the response subsides to near-normal rates shortly after irradiation is stopped. There is some indication that the magnitude of the respiratory rate stimulation response is based primarily upon dose rate. A comparison of softening and respiratory rate measurements indicates that the tissue softening is probably not directly related to the respiratory stimulation that occurs during gamma irradiation.

There are indications (Hannan, 1955; Morgan, 1955; U. S. Army Quartermaster Corps, 1957) that the storage life of some plant tissues can be lengthened by exposure to ionizing radiations in the 20–500 Kilorad (20,000–500,000 rad) dose range. The use of such radiation doses is now considered one of the promising applications of ionizing radiations for food preservation. From various tests in several laboratories, however, it appears that radiation in this dose range might at times also produce undesirable side effects. One of these is loss of the natural texture or firmness of the irradiated plant tissue.

Previous reports from this laboratory (Boyle *et al.*, 1957; Glegg *et al.*, 1956) have dealt with investigations of the radiation-induced tissue softening of apples, carrots, and beets. It seemed desirable to obtain, in addition, some information on the effects of radiation on a leafy vegetable. Lettuce was chosen. The first part of this paper reports on firmness (crispness or flexure) of gamma-irradiated lettuce leaves. Since the softening of (unirradiated) plant tissues appears generally to be linked either directly or indirectly to respirational activity, it seemed of interest to investigate the respirational activity of lettuce under the influence of gamma radiation to establish any relation that might exist between the respirational activity of lettuce so treated and the observed softening of the tissues. Accordingly, carbon dioxide evolution and oxygen consumption were measured during and following irradiation. The results are in the second part of this paper.

GAMMA-RADIATION-INDUCED SOFTENING OF LETTUCE TISSUE

Before embarking on this study, it was necessary to obtain a method for objective measurement of softening in lettuce leaves. Hannan's report (1955) stating that lettuce irradiated with 930 Kilorads of

[a] Approved by the Director of the New York State Agricultural Experiment Station, Geneva, as Journal Paper No. 1228, July 14, 1960. This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, QM Research and Engineering Command, U. S. Army, and has been assigned No. 2061 in the series of papers approved for publication.

cathode rays showed softening is often quoted in the literature (Morgan, 1955; U. S. Army Quartermaster Corps, 1957) together with a statement that softening occurs at even lower radiation doses (U. S. Quartermaster Food and Container Inst.). None of the experimental details of these tests have been published, and hence the instrumentation of these measurements could not be ascertained. Since a further search of the literature revealed no method suitable for our purpose, and since the instrument used in our studies of radiation-induced softening of apples, carrots, and beets could not be adapted to lettuce tissue, a new apparatus was needed. The instrument could be based on various physical properties of lettuce leaves. Since "crispness" is perhaps the most important texture characteristic of lettuce leaves, an instrument was constructed to measure the extent of flexure or bending resulting from the application of a defined force (weight).

Preparation of lettuce discs. The Iceberg-type lettuce used was bought at a local market on the day it arrived from the wholesaler. Until used it was stored at 34°F (2°C). One or two of the outer leaves were removed and discarded, and the samples were taken from the succeeding 4 or 5 leaves. For flexure measurements, discs 29.0 mm in diameter were cut with a cork borer so that neither the outer edge nor the thick base portion of the leaf were used. Disk thickness was measured with a micrometer gauge, 5 measurements per disc. Principally because of venation, average disc thickness varied from about 0.4 to over 2.0 mm. Only discs of 0.5–1.5 mm average thickness were used. At times the discs exhibited irregularities such as cupping, but only flat discs were used for these studies.

Method of flexure measurement. Fig. 1 is a diagrammatic drawing of the instrument constructed to measure the flexure of lettuce leaf discs. Its essential features are described in the caption to the figure.

Effect of lettuce disc thickness on flexure. The thickness measurements on a single lettuce-tissue disc were found to vary considerably, as does the average thickness of discs, even from the same leaf. The flexure caused by 100 g weight is less in a thick leaf than in a thin one, and therefore it was necessary to correct the flexure readings for disc thickness.

After measurements on 122 unirradiated lettuce samples had been accumulated, the regression line equation expressing the change of flexure with thickness was calculated. The equation was found to be $Y = 17.7x + 24.6$, with average thickness being 0.95 mm and average flexure 1.40 mm. The coefficient of correlation between the two measurements was 0.84 ($P < 0.01$). From the regression

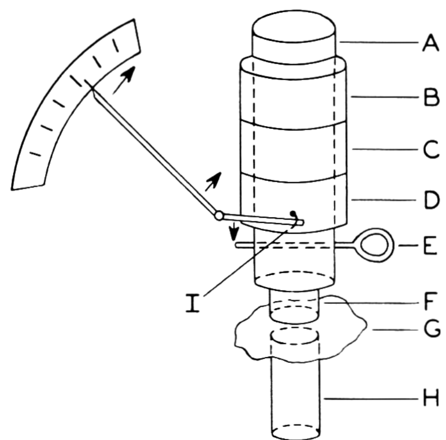

Fig. 1. Schematic drawing of the flexure measuring apparatus constructed to assess lettuce crispness. The disc of lettuce (G) is held by two supporting 12.5-mm-diameter steel rods (F and H). The weight-ring (D), inside diameter 21.3 mm and outside diameter 26 mm, and weighing 100 g, is allowed to slide down on tube A until it rests on the lettuce disc near the edge and away from the steel rods that hold the disc. The depression caused by the weight on the edges of the lettuce depends on its crispness, and this is measured by a pivoted indicator arm moved by hook (I) on the weight-ring. More weight can be put on the lettuce by adding other weight-rings (C and B). The indicator needle has an arm ratio of 1:5.6, and thus the flexure read on the scale is magnified 5.6 times. The reading is taken 15 seconds after the ring has been slowly lowered on the lettuce. Pin E holds up the weights while the lettuce is inserted and removed. Flexure measurements were made on both sides of the disc, and the values were then averaged.

line of flexure on thickness the formula $SF = F + [(T-1)(17.7)]/5.6$ was derived, in which SF is the standard flexure of a 1.0-mm-thick lettuce disc; F is the observed average flexure reading in mm from measurements on both sides of the disc; and T is the average measured thickness of the disc in mm. The formula corrects all flexure readings to that occurring on a disc of 1.00 mm average thickness. All flexure values given in this report are the standard flexure (SF) obtained with a ring-weight of 100 g and calculated by the above formula. Table 1 shows an example of a set of measurements and calculations.

Effects of hydration, dehydration, and rehydration on flexure. A major factor in lettuce crispness was found to be the water content of the tissue. To standardize the tissue for flexure measurement, full opportunity was first provided for maxi-

Table 1. Details of the determination of the standard flexure (SF) value for a sample (all values mm).

Sample	Thickness [a]		Flexure [b]			Standard flexure [c]
	Range	Av.	Side 1	Side 2	Av.	
1	0.54–0.98	0.70	12.6	9.8	11.2	1.04
2	0.60–1.20	0.88	8.0	8.0	8.0	1.04
3	0.79–1.22	1.02	6.0	7.8	6.9	1.30
4	0.50–0.86	0.65	14.0	13.0	13.5	1.29
5	0.71–1.39	1.12	6.0	8.0	7.0	1.64
6	0.42–0.88	0.63	10.2	12.4	11.3	0.82
7	1.00–1.82	1.42	4.0	5.4	4.7	2.19
8	0.70–0.96	0.80	11.0	16.8	13.9	1.84
9	0.67–1.32	0.88	8.0	7.4	7.7	0.98
10	0.55–1.56	1.07	8.4	1.6	5.0	1.13
Av.		0.92			8.92	1.33

[a] Five or more measurements made.
[b] The flexure measurement reading is magnified 5.6 × by the indicator arm ratio on the flexure tester.
[c] Corrected by formula (including division by 5.6) to give average thickness-corrected flexure (SF).

mum hydration. It was found that lettuce discs attain their maximum crispness (as measured by flexure) after one hour's soaking in water at 77°F (25.0°C). The value appears to remain constant during the next 4 hr. In all work reported here a 2-hour hydration period was used immediately before flexure measurement.

Effect of gamma radiation on the flexure of lettuce discs. Lettuce discs were packaged in Saran film (10 to a package) and irradiated at the University of Rochester Cobalt[60] source. The irradiation period used was about 18 hours, and the dosage was controlled by placing the samples at different distances from the source. Small plastic cups with tight-fitting plastic lids were also used for irradiating lettuce discs. The lettuce discs were placed with their plane surface at right angles to the radiation beam. The physical arrangements used in the irradiation have been described (Glegg *et al.*, 1956). The flexure measurements made on irradiated lettuce are summarized in Table 2.

The slight flexure change in lettuce discs irradiated with 600 Kilorad was not statistically significant, because of the high variability of individual measurements. Exposure to 1,000 Kilorad caused an increase in the flexure value, and these lettuce discs also showed visible radiation damage. They also deteriorated faster in terms of both flexure and appearance during post-irradiation holding at 77°F (25°C) than did the unirradiated control samples. In most instances the lettuce discs irradiated with around 100 Kilorad kept better than the control samples, but for some unknown reason this effect could not be reproduced consistently.

The dose of 320 Kilorad caused an apparent firming of the lettuce discs. A statistical comparison of the 6 control measurements with the 6 measurements at the 320-Kilorad level indicated a significant difference between the two groups at the 99% confidence level.

EFFECTS OF GAMMA RADIATION ON THE RESPIRATORY ACTIVITY OF LETTUCE LEAVES

Sussman (1953) found a transient increase in the carbon dioxide evolution and oxygen consumption rate of dormant potato tubers following irradiation with gamma rays in the kilorep dose range. Similar effects of irradiation upon higher-plant carbon dioxide production have been reported by other workers (Gustafson *et al.*, 1957; Smock and Sparrow, 1957) but none of these investigations included during-irradiation measurements or lettuce. In the present study, carbon dioxide production and oxygen uptake measurements were used as an indication of respiratory activity.

During-irradiation gas exchange. The design of the irradiation source used in these studies, referred to earlier (Glegg *et al.*, 1956), rendered impractical continuous during-irradiation respiratory activity measurement. With the exception noted below, carbon dioxide production and oxygen consumption rates were measured over the whole period of irradiation (about 18 hours). Used for this purpose were polyethylene boxes of about 300-ml capacity with closely fitting lids. For the carbon dioxide measurements, a small plastic ring was fixed to the inside bottom of the box to sup-

port a 5-ml Pyrex beaker containing 2 ml of 1N aqueous NaOH and a small filter-paper wick. The inner lip of the beaker was lightly coated with Vaseline to eliminate creeping of the alkali solution. For the oxygen measurements, each box was provided with a rubber ampoule stopper permitting insertion of a hypodermic needle for sampling the inclosed atmosphere.

The lettuce leaf samples were weighed and placed in the box. Immediately before irradiation the small beakers were charged with alkali and the lid placed onto the box, sealed with Vaseline, and secured with Cellophane tape. No attempt was made to modify the atmospheric composition at the start of the experiment from that of air. Following irradiation, a sample of gas was taken through the ampoule stopper for oxygen analysis by the Van Slyke manometric gas analysis apparatus. The oxygen content of the atmosphere in the plastic container at the conclusion of the irradiation was never less than 17%, a level well above that at which it becomes limiting for respiratory activity. The small beaker and its contents were placed in a rubber-stoppered wide-mouthed Erlenmeyer flask containing 50 ml of carbon dioxide-free water. Carbonate was then determined by the Hopkins modification of the barium precipitation method (Hopkins, 1924). The procedure involved the addition of 10 ml of 20% aqueous barium chloride and 10 ml of 95% ethanol, followed by titration with 0.1N HCl using phenolphthalein indicator. Appropriate control samples were included in all determinations. Both carbon dioxide evolution and oxygen consumption were calculated in terms of mg per kg fresh weight per hour.

Table 2. Effect of gamma radiation on lettuce disc leaves[a] (all values mm).

Dose (Kilorads)	Date of irradiation (1958)	Av. thickness	Measured flexure	Standard flexure	Av. standard flexure for radiation level
None	6/18	0.95	7.52	1.18	
None	6/18	1.11	4.75	1.19	
None	6/27	0.88	9.14	1.39	
None	7/25	0.98	8.60	1.32	
None	8/6	1.10	5.61	1.31	1.28
22	6/27	0.91	8.42	1.20	1.20
44	6/27	0.82	7.43	0.76	
44	6/27	1.00	6.11	1.08	0.92
90	6/17	0.94	8.00	1.24	
90	6/17	1.01	7.39	1.34	
90	6/27	0.92	8.92	1.30	
90	7/25	0.93	8.23	1.26	1.29
140	8/6	1.12	5.38	1.36	1.36
175	6/27	0.84	8.43	0.99	
175	6/27	0.84	10.03	1.17	1.08
320	6/17	0.97	5.70	0.97	
320	6/17	0.96	6.80	1.09	
320	6/27	0.69	12.19	1.01	
320	6/27	0.87	9.38	1.17	
320	7/25	0.80	8.79	0.94	
320	8/6	1.00	6.51	1.18	1.06
600	6/17	0.99	6.70	1.18	
600	6/17	0.99	8.44	1.49	
600	7/25	0.90	9.98	1.45	
600	8/6	1.15	5.58	1.47	1.40
1,000	6/27	0.84	20.65	2.77	
1,000	6/27	0.84	20.21	3.01	
1,000	7/25	0.97	9.80	1.55	2.48

[a] All values are the averages of the results obtained on 10 or more lettuce discs.

Post-irradiation gas exchange. Post-irradiation carbon dioxide evolution was measured in small chambers with a moving stream of moist carbon dioxide-free air. Nine such chambers, submerged in a water bath controlled at 25.0° ± 1°C, were used in these experiments. A modification of the carbon dioxide trapping method of Truog (1915) was used. The moistened carbon dioxide-free air stream is drawn over the tissue and then into an adsorption tower containing 0.02*N* NaOH. As shown in Fig. 2, the rate of flow of each unit was

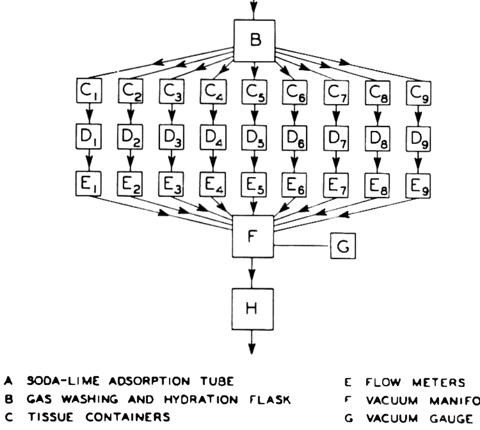

Fig. 2. Diagram of the nine-chamber respiratory rate measuring apparatus.

measured and controlled by a No. 2001-V (Size 2) Brooks-Mite Indicating Variable Flow-meter. The determinations started about two hours after irradiation, and the adsorption period was usually 90 minutes. The determinations consisted of 3 or 4 successive adsorption periods, using a new adsorption tower each time. The used towers were washed out with successive portions of carbon dioxide-free distilled water, and the sample titrated with 0.02*N* HCl. The results for the successive 90-minute periods were averaged. A flow rate of 6.9 L of air per hour, found satisfactory for the size of samples, was carefully maintained throughout the tests.

Intensity of light in the laboratory was found to exert no effect on carbon dioxide evolution rate of the lettuce samples. The rate could be influenced significantly, however, by placing a 100-watt incandescent light bulb near the respiration chamber. Care was therefore taken to avoid exposure to light of sufficient intensity to affect carbon dioxide evolution.

Successive leaves obtained from a lettuce head after removal of the two outermost leaves were found to vary little in carbon dioxide production. Leaves from different heads of similar history were similar in respiratory behavior, but storage periods extending beyond a week lowered the rate of carbon dioxide output. Only freshly purchased lettuce was used, and all samples within one experiment were taken from the same head. Crushing or visible microbial infection increased the rate of carbon dioxide production, and therefore only healthy leaves were used. In all samples the large midvein area in the basal portion of the leaf was removed by cutting with a sharp knife. The remainder of the leaf was divided into sections of 6–8 g, with a minimum amount of cutting.

Dehydration of lettuce leaf tissue to the point of incipient wilting significantly accelerated respiratory activity. For example, removal of 40% of fresh weight by exposure to dry air resulted in approximately 50% increase of the rate of carbon dioxide evolution as calculated on the original-weight basis. During these experiments the loss of moisture from the leaves was found to be negligible.

Effects of gamma radiation on lettuce respiration. The during-irradiation carbon dioxide evolution and oxygen consumption of lettuce were measured in the plastic boxes with radiation doses between 2 and 1,000 Kilorad. As shown in Fig. 3,

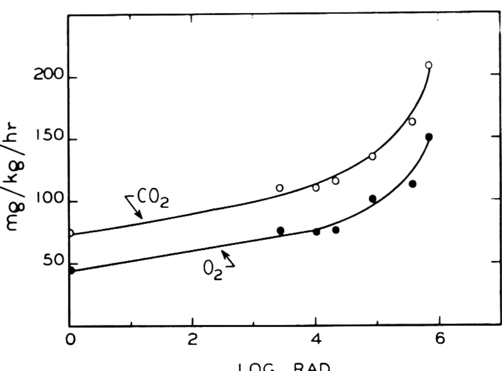

Fig. 3. Oxygen consumption and carbon dioxide evolution from lettuce leaves during an 18-hour period of irradiation.

both carbon dioxide evolution and oxygen consumption increased upon irradiation. The effect seems to be a gradual respiratory increase with increasing radiation dose. The shapes of the curves for both carbon dioxide evolution and oxygen consumption are similar. The carbon dioxide production–oxygen consumption quotient (RQ) decreased slightly as a result of irradiation, but all RQ values were between 1.2 and 1.6.

Table 3. Effect of gamma radiation on lettuce respiration when measured for the whole 18-hour irradiation period or for the first and second 9-hour periods separately.

Rate (Kilorad/hr)	Carbon dioxide production and oxygen consumption [a]							
	First 9 hr		Second 9 hr		Total 2		Uninterrupted 18 hr	
	CO_2	O_2	CO_2	O_2	CO_2	O_2	CO_2	O_2
None	91	60	81	73	86	67	90	56
1.13	106	65	88	62	97	64	101	71
4.43	112	92	95	67	104	80	111	72
16.2	145	104	135	97	140	101	137	101

[a] As mg per kg fresh weight per hour.

The during-irradiation experiments indicate the total results of the events taking place during the 18-hour period of irradiation during which the given dose was accumulated. It was impractical to determine the gas exchange of lettuce tissue under irradiation in the manner done in the post-irradiation measurements, but in some experiments measurements were made twice during the 18-hour irradiation period. The results of one such experiment (Table 3) indicate slightly higher carbon dioxide production and oxygen consumption rates during the first 9 hours of irradiation than during the second 9 hours. The average values for the 18 hours were similar, however, whether determined in one 18-hour or two consecutive 9-hour periods.

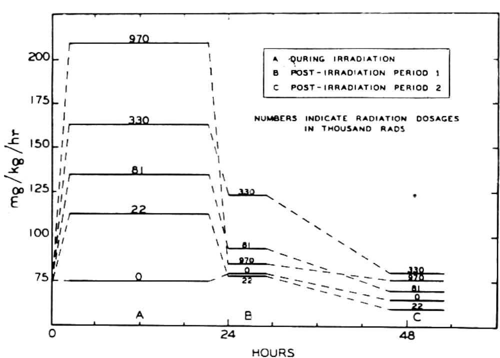

Fig. 4. Carbon dioxide evolution from lettuce leaves during and following irradiation.

Fig. 4 shows the carbon dioxide evolution of irradiated lettuce samples during and after irradiation. A comparison of the marked acceleration of carbon dioxide production during the irradiation with that during the post-irradiation measurements indicated that the radiation effect is relatively short-lived. With a radiation dose of 3 Kilorad, carbon dioxide returns to normal and slightly below within a few hours of the exposure. The sample irradiated with 300 Kilorad remained significantly higher than the control throughout the test period. Lettuce irradiated with 1,000 Kilorad responded most strongly during irradiation, and the carbon dioxide production of these samples also decreased more rapidly after termination of the irradiation than did lettuce exposed to lower doses. However, lettuce samples exposed to 1,000 Kilorad were obviously damaged, appeared yellow and "water-soaked," and deteriorated rapidly after irradiation. Twenty-four hours after irradiation there was little difference in carbon dioxide production among the samples that received different radiation doses.

No data are presented here for the oxygen consumption of lettuce leaves during the post-irradiation period. Enough information has been collected to show that oxygen consumption is parallel to carbon dioxide production in the manner shown for the during-irradiation period (Fig. 3).

DISCUSSION

Whether a definite "threshold dose" (TD) occurs in the radiation-induced softening of plant tissues is still unsettled. Such a TD might mean either the minimum dose where the radiation effect first occurs or represent the extent of change that first becomes measurable. Be this as it may, in our previous work on this subject we found that such TD values have meaning and reproducibility (Boyle *et al.*, 1957; Glegg *et al.*, 1956). Therefore, a brief discussion seems warranted of the present data in relation to a possible TD of the gamma-radiation-induced softening of lettuce tissue.

The flexure measurements used in following the softening of lettuce tissue are different from the breaking-strength measurements used in our studies of radiation-induced softening of storage-type tissues of apples, carrots, and beets. Nevertheless, it is of interest to compare the radiation-

induced softening of these various tissues both from the standpoint of TD and with regards to the extent of softening at higher doses.

On account of various limitations, the radiation dose range that we could apply in the present work included only one dose (1,000 Kilorad) where obvious softening of the lettuce occurred. At the next lower level, 600 Kilorad, there was no significant softening of the lettuce. Thus the TD of the softening of lettuce is somewhere between 600 and 1,000 Kilorad. Apples and carrots showed respective TD's of 32 and 109 Kilorad (Boyle *et al.*, 1957). The radiation-induced softening TD of beets is around 300 Kilorad (Glegg *et al.*, 1956), still definitely lower than the 600-Kilorad minimum value found for lettuce. Even with the use of different principles of measurement and with the large experimental variations, the results indicate that lettuce tissue is more resistant to gamma radiation than are apple, carrot, and beet tissues, and that the former has a significantly higher TD value.

The maximum softening measurable by our method is a flexure of about 9.0 mm. Since, with use of the standard 100-g weight, the control (unirradiated) lettuce discs showed an average flexure of 1.2 mm, the maximum possible change was 7.8 mm. The difference between the flexure of the controls and of the 1,000-Kilorad samples was only 1.3 mm, or 17% expressed on the basis of the maximum measurable range of softening. At this dose (and using a different method of measurement), apples and carrots showed respective softening of 70% and 50%.

The results reported here for the respiratory gas exchange of irradiated lettuce leaves are similar to those reported by other workers for other irradiated tissues (Gustafson *et al.*, 1957; Sussman, 1953). During irradiation, oxygen uptake and carbon dioxide production seem to be stimulated in a parallel manner. The stimulation is quite transitory, however, returning to near-normal rates soon after irradiation.

Irradiating lettuce leaf tissue in an interrupted manner gave some interesting results. The similarity of oxygen consumption and carbon dioxide production rates during two consecutive 9-hour periods of irradiation indicates that radiation-induced increase in respiratory activity does not depend on the total dose absorbed by the tissue, but rather on the dose rate. If the activation depended on total dose, then the second 9-hour period should have shown more activation than the first 9-hour period. The total dose at the end of the 18-hour period was twice that at the end of the first 9-hour period. The data on hand do not answer the question whether the activation is a continuous function or occurs as an initial surge of activity followed by lower rates. From the data obtained for the two 9-hour periods, however, such a surge seems unlikely.

One may conclude that the major portion of the radiation-induced tissue softening is not a direct result of respiratory acceleration but rather a separate and probably more direct response. This interpretation is supported by the gradual increase of the respiratory response at radiation doses where no tissue softening could be demonstrated. The respiratory activation was quite definite at 22 Kilorads, whereas measurable tissue softening did not occur until the dose was in excess of 600 Kilorad. This lack of direct relationship between respiratory stimulation and softening, and the close relation observed between softening and changes in the pectic constituents of apples, carrots, and beets (Kertesz *et al.*, 1961), seem to indicate that any softening brought about by physiological response to radiation is minor in comparison to the chemical changes leading to tissue softening. Even so, the present data do not rule out the possibility that some of the softening above the softening TD of the tissue might be associated with the increased respiratory activity.

Acknowledgment

We are indebted to Dr. L. W. Tuttle and the Atomic Energy Project, University of Rochester School of Medicine and Dentistry, Rochester, N. Y., for their continued cooperation in allowing the use of their Co^{60} radiation facility for these experiments.

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Browning of Sugar Solutions. VI. Isolation and Characterization of the Brown Pigment in Maple Sirup[a]

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SUMMARY

The colorant of maple sirup has been isolated using ion exchange resins and purified by dialysis. The purified pigment had an empirical formula of $C_{15}H_{27}O_{12}N$.

INTRODUCTION

The value of maple sirup resides strictly in its maple flavor. This attribute, however, cannot be measured objectively, and maple sirup is graded on its color. The lighter-colored sirups have less total flavor, but contain a more true, distinctive maple flavor than darker ones, and therefore command a higher market price. (The light-colored sirup is especially important in the making of maple confections.) Thus, the maple sirup producer must control the factors that cause the development of color in his product. Much excellent work has been done on the causes and control of color formation in sugar solutions and other food products. Hodge (1953), Liggett and Deitz (1954), and Zerban (1947) have published reviews in the field.

The color of maple sirup, as well as the flavor, is developed during the atmospheric boiling process used to concentrate maple sap to sirup. This was first demonstrated by Findlay and Snell (1935), who produced colorless sirup by vacuum-distilling maple sap, and was later confirmed at this laboratory by freeze drying. Also, the effect of pH on color development during the evaporation of sap was studied by Hayward and Pederson (1946) and Bois and Dugal (1940). Edson *et al.* (1912) observed that sap heavily contaminated with microorganisms produced dark-colored sirup. Hayward and Pederson (1946) noted that the invert sugar content of the sap affected the color of the sirup. Maple sap with no invert in it produces very light sirups. Holgate (1950) confirmed these findings. These observations correlate with data of Naghski and Willits *et al.* (1952) which also show that the sterile sap as it comes from the tree is fermented by adventitious organisms that produce different amounts of invert sugar. These reducing sugars are the main source of color in maple sirup. This information has permitted better control of the operations of sirup production, substantially increasing the amount of the lighter grades of maple sirup made. Nevertheless, knowledge of the mechanism of the color formation and of the chemical composition of the color bodies is still incomplete. Because the spectral characteristics of the color of maple products are similar to those of other food products and to sugar solutions, such information would be of value, then, not only to the maple industry but also to the sugar and food industry as a whole. Consequently, this study was initiated to determine the chemical composition of the colorant in maple sirups. Present information indicates that maple colorant is polymeric in nature, and further, in light-colored sirup may be totally different from that in dark-colored ones. This paper presents the results of work on the isolation and characterization of the colorant in light-colored sirups.

EXPERIMENTAL

Isolation of the Maple Sirup Colorant

Four hundred grams of Duolite A-4 anion exchange resin was washed free of suspended solids and packed as a wet slurry 78 cm deep in a glass column of 5.5 × 150 cm. A glass siphon tube

[a] Presented before the 20th annual meeting of the Institute of Food Technologists, San Francisco, California, May, 1960.

[b] Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture.

was connected to the bottom of the column through a ball-and-socket joint. This served to maintain a constant level of solution 3 cm above the resin. Flow rate through the resin was controlled with a glass stopcock fitted to the top portion of the siphon tube.

The resin was put in the hydroxyl form by passing 18 L of 5% aqueous sodium hydroxide through the column. After the resin was washed free of excess alkali with distilled water, it was placed in the chloride form with 16 L of 5% sodium chloride solution. The resin was freed of excess chloride by washing again with distilled water.

A medium-amber grade of maple sirup, previously extracted with chloroform to remove maple flavor, was diluted with an equal volume of water and passed through the column. Eight liters of sirup saturated the resin with color, as indicated by the appearance of color in the effluent. At this point the flow of sirup through the column was stopped and the resin was washed free of sugar with distilled water.

The adsorbed maple color, free of sugar, was recovered from the column with 10 L of 5% sodium chloride. The elution with 5% sodium chloride was continued to regenerate the column as indicated by a clear eluate. Then the column was washed free of chloride and was ready for the passage of more sirup.

The brown maple colorant eluted from the ion exchange column was concentrated and freed of sodium chloride with a modification of the dialyzing concentrator described by Smith and Stevens (1942). A diagrammatic sketch of this unit is shown in Fig. 1. With a 250-ml. capacity, the

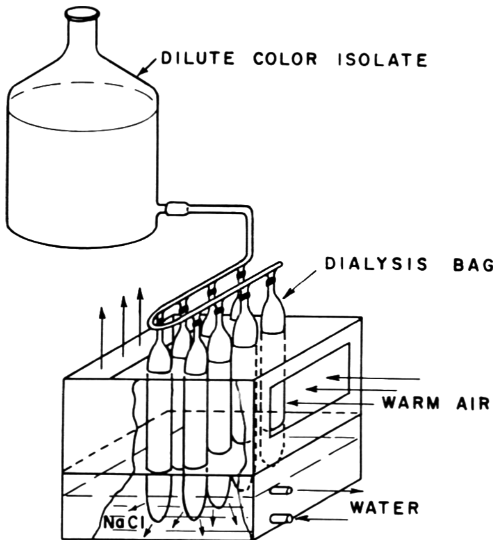

Fig. 1. Diagram of dialyzing concentrator.

apparatus evaporated water at the rate of 2 L per hour. The viscous dark-colored sirup obtained from 4 L of column effluent had a pH of 6.6. This color concentrate, when dewatered by lyophilization, yielded a dark-brown solid, which was further dried under vacuum for 4 hours (0.1 mm) at 40°C. This material proved to be high in ash indicating that subsequent isolates should be further purified. Therefore, another color concentrate from the dialysis-evaporation unit was adjusted to a pH of 3 with hydrochloric acid and dialyzed against distilled water until free of chloride. The dried colorant from this additional treatment was low in ash. By this procedure 32 L of maple sirup gave 0.12 g (3–4 ppm) of dry colorant.

Characterization of the Isolated Pigment

1. *Elemental Analyses.* Two different samples of the isolated pigments were analyzed for carbon, hydrogen, nitrogen, and ash. The composition of the colorant from the dialysis concentrator before acidification was compared to that after acidification. The values are recorded in Table 1.

Table 1. Elemental composition of two samples of colorant from maple sirup.

Determination	Sample [a] A [b]	Sample [a] B [c]
Carbon	48.5	47.8
Hydrogen	6.8	6.0
Nitrogen	3.6	3.1
Oxygen [d]	41.1	43.1
Ash	20.5	4.2

[a] Salt-free basis.
[b] From neutral salt solution.
[c] From acidified salt solution.
[d] By difference.

2. *Infrared Absorption Data.* Infrared absorption curves were obtained for the isolates from the neutral and acidified dialysis concentrates (Fig. 2). The pigments had characteristics of a carbohydrate, as indicated by strong hydroxyl and weak CH bands. The hydroxyl groups could be primary or secondary. No evidence of a carbonyl group was present in the neutral (unacidified) concentrate.

Strong absorption bands indicated the presence of carboxylate ion as an inorganic salt. Reduction of the ash by acidification and further dialysis weakened the carboxylate band. However, there was no evidence of a free carboxyl group building up, or of any other group that could form a salt. This is indirect evidence that the ash came from sodium salt of one of the organic acids, probably the malic acid present in the isolate as an impurity

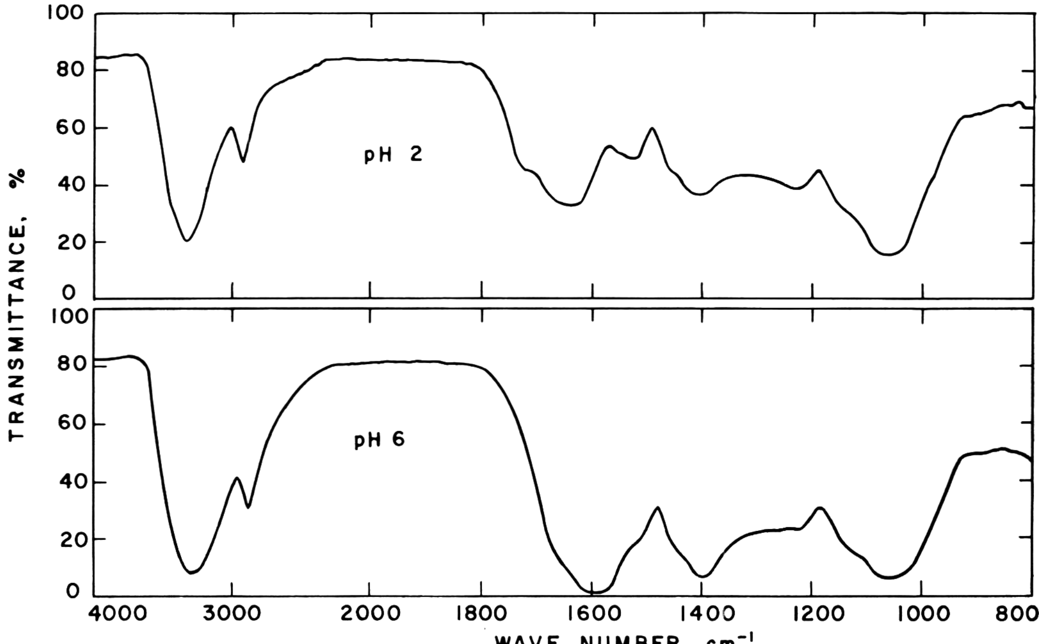

Fig. 2. Infrared curves of maple sirup colorant isolates before (pH 6) and after adjustment to pH 2 with acid.

3. *Ultraviolet Absorption.* The ultraviolet absorption curve at 400 to 225 mμ of the colorant from the neutral dialysis concentrate showed little character except a rapid increase in absorption as the wave length of light decreased, especially below 250 mμ. The acid treatment did not change this significantly.

DISCUSSION OF RESULTS

Several interesting properties of the colorant of maple sirup may be cited from the data:

1) Since the colorant can be separated from the sugars of the maple sirup with an anion exchange resin, it appears to be ionic and to carry a negative charge.

2) Elemental analysis indicates the colorant to have a composition similar to a carbohydrate containing nitrogen. The manner in which the nitrogen is incorporated in the colorant will be the subject of future investigations. Since the nitrogen content of maple sap is extremely small, about 10 ppm, it can be involved directly in forming only a limited amount of color. This would be more than enough to account for the small amount of colorant in a light-colored sirup (3–4 ppm isolated from the sirup used in this study). But maple sirup with continued boiling turns darker and darker. This latter color could be formed by a caramelization reaction, which may or may not involve nitrogen. Thus, two mechanisms could be responsible for the color of maple sirup.

3) The infrared absorption curves of the two colorants gave evidence that the pigment was carbohydrate in nature: absorption bands in the OH region were strong relative to the CH band.

4) Earlier work on isolating the colorant with ion exchange resins showed that the pigment is not homogeneous in composition. Maple sirup colorant that adsorbed on an anion exchange resin in the hydroxyl form could be separated into fractions by a series of increasingly alkaline eluting solutions—sodium chloride, sodium carbonate, and sodium hydroxide.

5) The maple colorant described here is quite similar to that isolated by W. W. Binkley, New York Sugar Trade Laboratory, by dialysis alone from cane final molasses (Binkley, 1957). From elemental analysis of the isolate he derived an empirical formula $C_{17}H_{26\text{-}27}O_{10}N$. The formula for the pigment in the maple sirup was found

by us to be $C_{18}H_{27}O_{12}N$. Thus, the compositions of the two colorants are very similar.

Acknowledgments

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Citrus Flavor. Volatile Constituents of the Essential Oil of the Orange (*Citrus sinensis*)[a, b]

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SUMMARY

Analysis by gas-liquid chromatography (GLC) of the essential oil from California Valencia oranges revealed over 50 components. A preliminary separation of the intact oil into two major fractions, the terpenes and terpenoids, was made by liquid-solid chromatographic techniques. Examination of the terpene fraction by GLC revealed 15 constituents, of which a number were tentatively identified. The terpenoid fraction contained some 37 constituents, many of which were tentatively identified. These techniques increased resolution and permitted tentative identification of some 14 compounds not hitherto reported in Valencia orange oil. These compounds are: 3-hepten-1-ol; *n*-octyl acetate; *n*-undecanal; bornyl acetate; isopulegol; *n*-decyl acetate; borneol; citronellol; neral; geranyl acetate; α-pinene; α-phellandrene; γ-terpinene; and *p*-cymene.

The constituents were tentatively identified by calculating relative corrected retention volumes and comparing these with values for known compounds. Two stationary liquid phases used yielded data that lend credence to the tentative assignment of identities of the constituents isolated. In addition, infrared examination of some collected fractions made possible partial identification of the compounds.

The nature of the characteristic flavor of the orange has long intrigued researchers. It is known that the flavor of citrus products, e.g., juices, is derived largely from the essential oil in the juice sacs and expressed from the oil sacs of the flavedo of the fruit (Hall and Wilson, 1925; Itoga, 1959; Kefford, 1955, 1959; Kirchner and Miller, 1952; Naves, 1932). Indeed, Kefford (1955) flatly stated "The characteristic aroma and flavours of citrus fruits are due chiefly to aldehydes and esters in the peel oils."

Itoga (1959) attempted to correlate citrus flavor with measurements of the physical properties (absorbance, colloidal turbidity, fluorescence) of some of the flavor components of the essential oils, but met with no notable success.

A number of workers have approached the flavor problem by examining the essential oils obtained from citrus. As early as 1900 Stephan (1900) reported on his findings as to the composition of orange oil. Poore (1932) made the first comprehensive examination of California orange oil and described a large number of its constituents. Naves (1932, 1947), and later Benezet and Igolen (1946), examined French Guinea orange oil and sweet orange oil (Naves, 1947), and both groups further elucidated the composition of the aldehyde fraction. Nelson and Mottern (1934) made a detailed examination of the citral content of Florida Valencia orange oil, and Guenther and Grimm (1938) later conducted a similar study on California orange oils.

Schweisheimer (1955) attempted to find what he described as the "odor principle" in orange skins. Kirchner and Miller (1957) and Hall and Wilson (1925) examined volatile materials obtained by distillation of large volumes of California orange juice.

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As new techniques and methods for analysis develop, knowledge of the chemical composition of the essential oils increases at a steady rate. With the advent of the powerful technique of gas-liquid chromatography (GLC) (Keulemans, 1957) and its subsequent application to study of the essential oils (Bernhard, 1957, 1958, 1960a; Calvarano, 1957; Clark and Bernhard, 1960a, b) an interesting avenue for flavor research opened.

Employing techniques developed from a number of his earlier studies on lemon oils (Bernhard, 1957, 1958, 1960a; Clark and Bernhard, 1960a, b) the author investigated the components of orange oils.

The success of such a program is largely dependent upon proper choice of the stationary liquid phase or phases. Careful examination of over 40 high-boiling liquids (Bernhard, 1960b) revealed that the polyesters such as LAC-2-R446 (the adipate polyester of diethylene glycol partially cross-linked with pentaerythritol) (Lipsky and Landowne, 1958) and LAC-4-R777 (the succinate polyester of diethylene glycol) (Lipsky and Landowne, 1959) are well-suited for separating the components of the citrus oils by GLC.

In addition a preseparation of the major components into two fractions before gas chromatographic examination proved helpful. The arguments for this technique and its application to lemon oil were detailed by Clark and Bernhard (1960a, b; Dal Nogare and Bennett, 1958). Employing this convenient technique with orange oil, some 14 additional compounds were more clearly detected and evaluated.

EXPERIMENTAL

The apparatus used to separate the constituents of cold-pressed California orange oil and the procedure for the modified Kirchner and Miller deterpenation have been described in detail (Clark and Bernhard, 1960a; Dal Nogare and Bennett, 1958; Kirchner and Miller, 1952).

Columns were constructed of stainless-steel tubing, ¼ in. OD and 10 ft long. The support material used throughout this study was Sil-O-Cel C-22 diatomaceous earth firebrick (30–60 mesh). Fractions of this were sieved to size, further graded by sedimentation in water, and thoroughly dried in an oven at 125°C for 10 hours. The liquid phase was introduced by deposition from benzene solution, the packing then being heated in a stream of helium at 180°C for 12 hours. The materials were applied to the solid support in the amount of 20% (w/w).

The parameters of operation, i.e., temperature, flow rate, etc., accompany the figures and tables.

The orange-oil samples examined were cold-pressed California Valencia orange oils obtained from the Research Department of Sunkist Growers, Inc., Ontario, California. They were from last year's crop of fruit (February-March, 1959) and were so-called raw-oil samples (no waxes removed). A typical oil had the following physical properties:

Property	Value
Specific gravity at 25°/25°	0.843
Optical rotation at 25°	+96.5°
Refractive index at 20°	1.4732
Evaporation residue	3.3%
Aldehyde content, calculated as decanal (hydroxylamine method)	1.4%
Color	orange to reddish-orange

RESULTS AND DISCUSSION

Gas-liquid chromatograms of whole Valencia orange oils employing the stationary liquid phases described in this study reveal very little as to the total component picture of the oil. Some 18 peaks are evident (Fig. 1). Peaks 1 through 5 are extremely large and sharp, as are peaks 6 and 10, but the remaining 11 peaks are ill-defined.

Employing a stationary liquid phase of LAC-2-R446 (Lipsky and Landowne, 1958), 23 peaks were evident on the chromatogram (Fig. 1); 18 were numbered for identification; 5 small peaks near the origin of the chromatogram (between the air peak and peak 1) are not numbered. These peaks differ from sample to sample in both position (i.e., retention distance) and amount, and have not yet presented any consistent pattern. The numbered peaks generally differ from sample to sample only in amount. Thus the principal investigations and identifications were concerned with these components. Work is in progress on identification of the unnumbered peaks in various oil samples. The various peaks were identified by determining the corrected retention volumes (V°_{R}) (Ambrose *et al.*, 1958) of a series of known compounds, and comparing them with those for the unknown peaks. In addition, some of the components were iden-

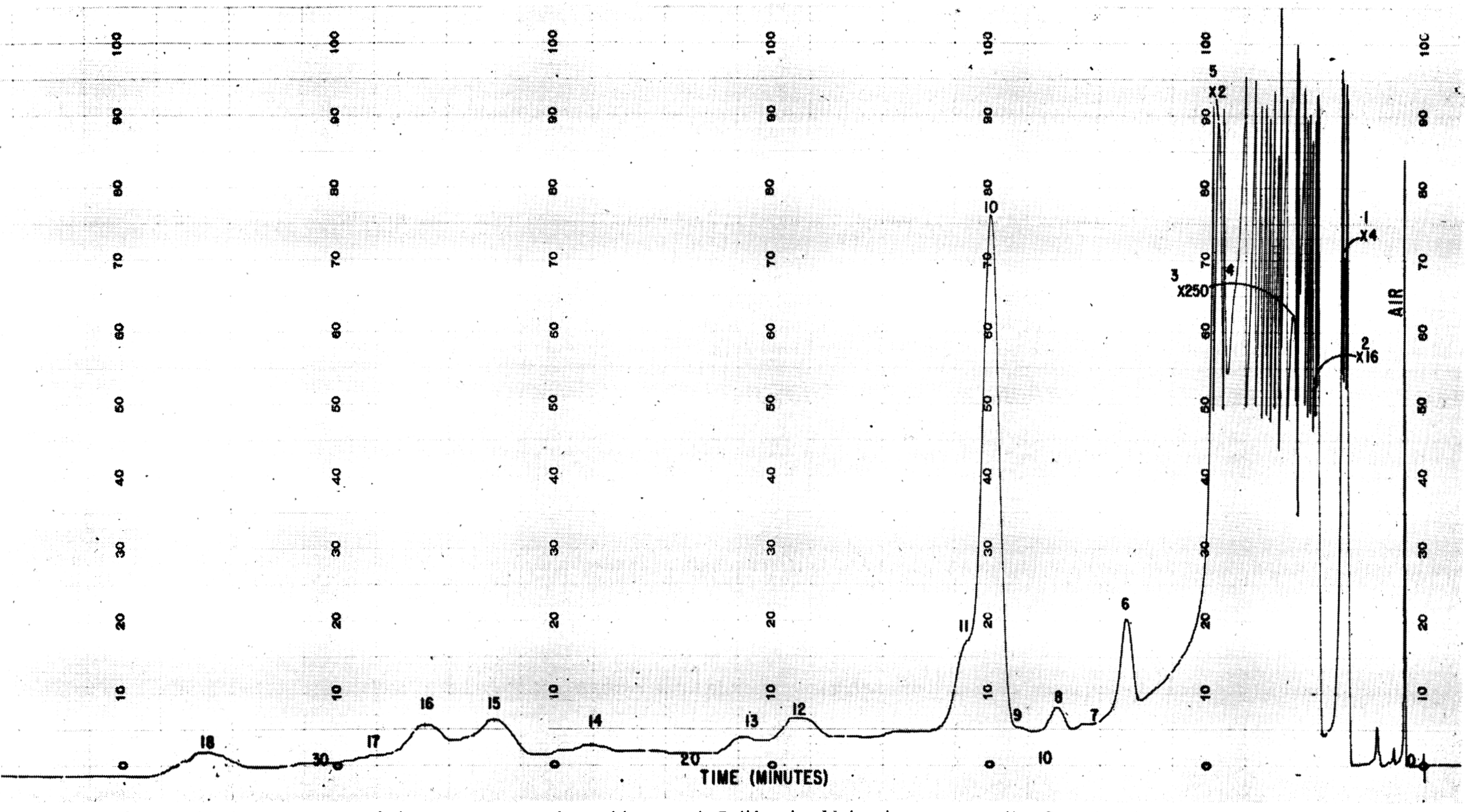

Fig. 1. Gas-liquid chromatogram of the components of a cold-pressed California Valencia orange oil. Sample size: 20 μl; temperature 150°C; helium flow rate: 90 ml/min; stationary phase: LAC-2-R446 on a support of Sil-O-Cel C-22 (30–60 mesh), 20% w/w; stainless-steel column 10 ft by ¼ in. OD; 1-mV recording potentiometer; chart speed: 30 in./hr. Peak identities are presented in Table 1.

tified by trapping the volatile components as they issued from the chromatograph and subjecting these fractions to infrared analysis. It was not possible to obtain unambiguous infrared spectra for all trapped fractions, since many samples were found to contain impurities from neighboring factions, or were gross mixtures of several components appearing in the same fraction. By these means a large number of the components were tentatively identified. The data (Table 1) are presented in the form of relative corrected retention volumes ($V°_R/V°_R$) (Bernhard, 1960a). It is well to note that it is experimentally impossible to discriminate between two or more compounds whose relative retention volumes differ by about 7% or less, since they will appear on the chromatogram as a single, united peak. A 10–15% difference in relative retention volumes will show peaks that are partially united, i.e., shoulders or doublets. Differences of 20% or greater are necessary for complete separation of zones or peaks under the conditions obtaining in these experiments (Bernhard, 1957; James, 1956).

Table 1. Relative corrected retention volumes of the components of a cold-pressed California Valencia orange oil. Stationary phase: LAC-2-R446.[a] (n-decanal = 1.00).

Peak	$V_R°/V_R°$ Unknown	Known	Compound
1	0.142	(0.148)	(α-pinene)
2	0.218	(0.227)	(myrcene)
3	0.270	(0.298)	(D-limonene)
4	0.410	.....	....
5	0.450	0.455	n-octanal
6	0.671	0.676	n-nonanal
7	0.761	(0.785)	(iso-amyl caproate)
8	0.841	0.840	n-octyl acetate
9	0.931	(0.965)	(furfural)
10	1.00	1.00	n-decanal; 1-octanol
11	1.06	1.06	linalool
12	1.47	1.43	citronellal
		1.47	n-undecanal
		1.50	isopulegol; bornyl acetate
13	1.60	1.61	geranyl formate
14	1.97	1.95	borneol
15	2.20	2.17	n-dodecanal
		2.23	1-decanol; terpinyl acetate
16	2.38	2.38	citronellol; neral
17	2.49	(2.43)	(geraniol)
18	2.90	2.89	citral

[a] Temperature: 150°C; helium flow rate: 90 ml/min.

To be well within the bounds of experimental error, an arbitrary limit of agreement not to exceed 2% was established. Compounds with values for relative corrected retention volume not agreeing within 2% of each other are enclosed in parentheses. In Table 1, peaks that differ by more than the 2% limit of agreement are: peak 1, α-pinene (4% difference); peak 2, myrcene (4% difference); peak 3, D-limonene (9.4% difference); peak 7, iso-amyl caproate (3.1% difference); peak 9, furfural (3.5% difference); and peak 17, geraniol (2.5% difference). Except for peak 3, these differences are well within the 7% limit found by James (1956) and Bernhard (1957). The limonene peak is so large (as compared to the other peaks) that some skewing takes place. This makes it very difficult to establish with precision the exact retention distance, and hence retention volume, of this component. That may account for the 9.4% difference in agreement with known values. Experiments made with low sample volumes of whole orange oil show good agreement between this unknown peak and authentic samples of D-limonene. Because of the low sample volume, most of the other peaks do not appear on the chromatogram; thus, the sample size shown (Fig. 1) was chosen to permit display of the maximum number of peaks without excessive column overload and subsequent distortion.

As a further check on identity, relative retention volumes were evaluated on a second stationary liquid phase, LAC-4-R777 (Lipsky and Landowne, 1959) (Table 2). The data supplied lend credence to the tentative identification of the constituents. With a liquid phase of LAC-4-R777, orange oil showed 20 peaks on the chromatogram; 15 are numbered for identification. As noted above, there were, near the origin of the chromatogram, 5 small peaks that were not numbered. These differed in both retention distance and amount from sample to sample, presenting no consistent pattern. Work is in progress to identify these substances. The unknown peaks were identified

Table 2. Relative corrected retention volumes of the components of a cold-pressed California Valencia orange oil. Stationary phase: LAC-4-R777.[a] (n-decanal = 1.00).

	V_R°/V_R°		
Peak	Unknown	Known	Compound
1	0.145	(0.136)	(α-pinene)
2	0.229	(0.217)	(β-pinene)
3	0.302	(0.294)	(D-limonene)
4	0.497	0.495	n-octanal
		0.500	n-hexanol
5	0.704	0.701	n-nonanal
6	0.844	0.849	n-octyl acetate
7	1.00	1.00	n-decanal
8	1.18	(1.15)	(linalool)
		1.18	linalyl acetate
9	1.26	.....	....
10	1.40	1.41	linalyl propionate; n-undecanal
11	2.02	2.01	n-dodecanal
12	2.29	2.31	terpinyl acetate
		(2.36)	(borneol)
13	2.41	2.41	geraniol; α-terpineol
		(2.46)	(citronellol)
14	2.70	2.74	geranyl acetate
15	3.18	3.14	neral
		3.22	citral

[a] Temperature: 150°C; helium flow rate: 90 ml/min.

on the basis of agreement of relative corrected retention volumes with values for known compounds. In addition, infrared spectra were obtained from as many fractions as could be satisfactorily trapped and examined. Data are presented in the form of relative corrected retention volumes (Table 2). The peaks differing by more than the 2% limit of agreement are: peak 1, α-pinene (6.6% difference): peak 2, β-pinene (5.5% difference); peak 3, D-limonene (2.7% difference); peak 8, linalool (2.6% difference); peak 12, borneol (3.0% difference); and peak 13, citronellol (2.1% difference). Once again, these differences are within the 7% limit discussed above (Bernhard, 1957; James, 1956). Skewing of the terpene peaks near the origin of the chromatogram doubtless accounts for the larger percentage differences between known and unknown relative corrected retention volumes in this region.

The use of an additional stationary liquid phase helps reinforce the assignment of tentative peak identities by the methods described above. Good correlation was achieved between both sets of relative corrected retention volume data obtained by the use of the two stationary liquid phases for the following compounds: α-pinene, D-limonene, n-octanal, n-nonanal, n-octyl acetate, n-decanal, linalool, n-undecanal, n-dodecanal, borneol, terpinyl acetate, citronellol, neral, geraniol, and citral.

Terpene fraction. The terpene fraction is composed mainly of compounds that are structural isomers of 1-methyl-4-isopropylcyclohexadienes. This fraction has the aroma typically associated with turpentine or "lighter fluid," possessing virtually none of the pleasant aroma characteristic of the orange. The terpene fractions, obtained as described above, were colorless oils of low density and viscosity. The terpenes from orange oil were quite stable when stored at −10°C in the absence of light, trace metals, and oxygen.

When the terpene fraction, obtained from the deterpenation procedure, was examined employing a stationary liquid phase of LAC-2-R446, 15 peaks were evident on the chromatogram (Fig. 2). Peaks 1 through 4 are due to some residual solvent remaining in the fraction (in this instance, commercial "hexanes" were used to elute the terpene fraction from the silicic acid adsorbent). It may be seen that peak 11, D-limonene, is the major component in this fraction. Data are presented in the form of relative corrected retention volumes (Table 3). Identification of the components was made as described above, using retention volume data and infrared spectral techniques. The peaks (Table 3) that differ by more than the 2% limit of agreement are: peak 5, α-pinene (5.3% difference); and peak 14, p-cymene (3.2% difference) Again these differences are well within the 7% limit discussed by Bernhard (1957) and James (1956).

When the terpene fraction was examined on the second stationary liquid phase, LAC-4-R777, only 7 peaks were evident. Data are presented as noted above (Table 4). None of the compounds identified exceeded the 2% limit of agreement. Correlation between the two sets of relative corrected retention

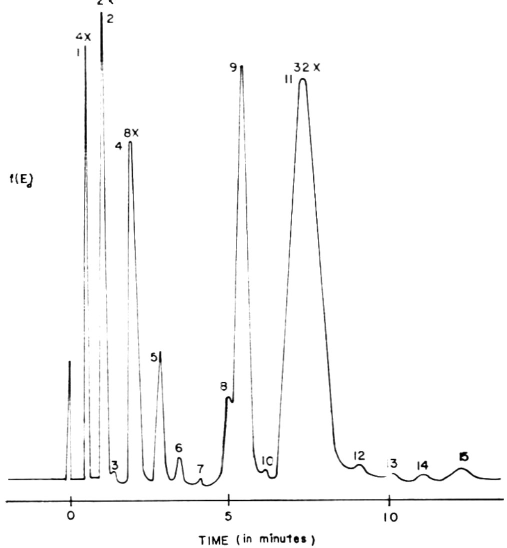

Fig. 2. Gas-liquid chromatogram of the components of the terpene hydrocarbon fraction of a cold-pressed California Valencia orange oil. Sample size: 10 μl; temperature: 100°C; helium flow rate: 145 ml/min; stationary phase: LAC-2-R446 on a support of Sil-O-Cel C-22 (30–60 mesh), 20% w/w; stainless-steel column 10 ft by ¼-in. OD; 1-mV recording potentiometer; chart speed: 30 in./hour. Peak identities are presented in Table 2.

Table 3. Relative corrected retention volumes of the components of the terpene fraction of a cold-pressed California Valencia orange oil. Stationary phase: LAC-2-R446.[a] (D-limonene = 1.00).

Peak	$V_R°/V_R°$ Unknown	Known	Compound
1	0.079	......	solvent
2	0.140	......	solvent
3	0.186	......	solvent
4	0.271	......	solvent
5	0.395	(0.375)	(α-pinene)[b]
6	0.477	......	....
7	0.559	......	....
8	0.699	......	....
9	0.756	0.758	myrcene[b]
10	0.838	0.837	α-phellandrene
11	1.00	1.00	D-limonene[b]
12	1.25	1.26	γ-terpinene
13	1.39	......	....
14	1.53	(1.58)	(p-cymene)[b]
15	1.70	......	....

[a] Temperature: 100°C; helium flow rate: 145 ml/min.
[b] Infrared spectrum for unknown compound agrees with spectrum for known compound.

volumes was excellent for the following: α-pinene, myrcene, D-limonene, and γ-terpinene. The deterpenation procedure yielded a fraction that permitted identification of at least three additional terpene hydrocarbons over those noted by examination of chromatograms of the whole or intact oil. These are: α-phellandrene, γ-terpinene, and p-cymene. The last compound is, in reality, not a true terpene hydrocarbon, but an aromatic hydrocarbon frequently found in the terpene fractions of citrus oils (Clark and Bernhard, 1960a). Since it appears in this fraction normally, it is included under the heading "terpene fraction."

Table 4. Relative corrected retention volumes of the components of the terpene fraction of a cold-pressed California Valencia orange oil. Stationary phase: LAC-4-R777.[a] (D-limonene = 1.00).

Peak	$V_R°/V_R°$ Unknown	Known	Compound
1	0.229	......	....
2	0.373	0.372	α-pinene
3	0.621	......	....
4	0.712	......	....
5	0.778	0.776	myrcene
6	1.00	1.00	D-limonene
7	1.27	1.25	γ-terpinene

[a] Temperautre: 100°C; helium flow rate: 145 ml/min.

Terpenoid fraction. This fraction, sometimes called the oxy fraction, comprises mainly those compounds that contain oxygen or bear oxygen-containing functional groups, e.g., esters, aldehydes, ketones, acids. It is this fraction that possesses most of the characteristic aroma typically associated with the orange. The color of the fraction ranges from pale-yellow to orange-red, and it has a higher viscosity than the terpene fraction. The terpenoids from orange oil have good stability, especially when kept in the dark, free of air (or oxygen), and rather cold (−10°C). Samples of these fractions kept in this laboratory in the dark and at −10°C for well over one year still have a fresh, pleasing aroma, and good color.

Samples of the terpenoid fraction examined, using a stationary liquid phase consisting of LAC-2-R-446, yielded chroma-

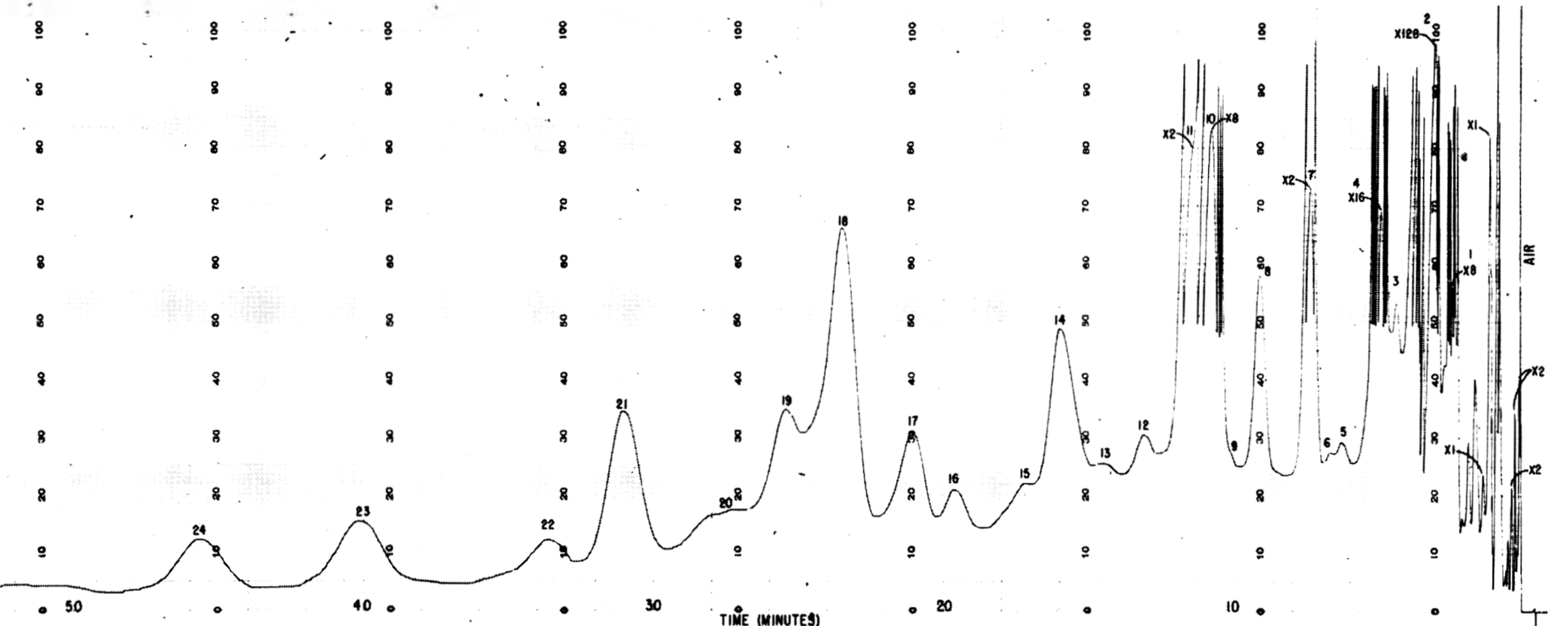

Fig. 3. Gas-liquid chromatogram of the components of the terpenoid fraction of a cold-pressed California Valencia orange oil. Sample size: 20 μl; temperature: 150°C; helium flow rate: 90 ml/min; stationary phase: LAC-2-R446 on a support of Sil-O-Cel C-22 (30–60 mesh), 20% w/w; stainless-steel column 10 ft by ¼ in. OD; 1-mV recording potentiometer; chart speed: 30 in./hr. Peak identities are presented in Table 3.

tograms showing 36 distinct peaks (Fig. 3). Of these, 24 are numbered for identification. Again, some 12 minor peaks near the origin show no consistent pattern from sample to sample as to retention distance and amount. Obviously, one of these peaks is due to some residual solvent, ethanol, used to elute the terpenoids from the silicic acid. This appears as the first large peak after the air peak. Data are presented in the form of relative corrected retention volumes (Table 5). It may be noted that some of the terpene hydrocarbons are still present in this fraction. However, it does not appear essential that they be completely removed in order to raise the concentration level of the oxy compounds and thus achieve detection. The components were identified as described above, using infrared spectra when possible, and comparing relative corrected retention volume data. The peaks (Table 5) that differ by more than the 2% limit of agreement are: Peak 1, myrcene (2.2% difference); peak 2, D-limonene (6.7% difference); peak 12, citronellyl acetate (2.5% difference); and peak 23, *trans*-carveol (3.6% difference). Once again, these values are within the 7% limit discussed above.

Table 5. Relative corrected retention volumes of the components of the terpenoid fraction of a cold-pressed California Valencia orange oil. Stationary phase: LAC-2-R446.[a] (*n*-decanal = 1.00).

Peak	V_R Unknown	V_R' Known	Compound
1	0.222	(0.227)	(myrcene)
2	0.278	(0.298)	(D-limonene)
3	0.404	.....	
4	0.456	0.455	*n*-octanal
5	0.578	0.571	methyl heptenone
6	0.615	0.621	linalyl acetate
7	0.685	0.676	*n*-nonanal
		0.689	3-hepten-1-ol
8	0.844	0.840	*n*-octyl acetate
9	0.945	0.964	furfural
10	1.00	1.00	*n*-decanal
11	1.06	1.06	linalool
12	1.21	(1.18)	(citronellyl acetate)
13	1.34	.....	
14	1.49	1.47	*n*-undecanal
		1.50	isopulegol; bornyl acetate
15	1.61	1.61	geranyl formate
16	1.83	1.83	decyl acetate
17	1.97	1.95	borneol
18	2.20	2.17	*n*-dodecanal
		2.23	1-decanol; terpinyl acetate
19	2.38	2.38	citronellol; neral
20	2.62	2.61	geranyl acetate
21	2.90	2.89	citral
22	3.15	3.10	D-carvone
23	3.76	(3.63)	(*trans*-carveol)
24	4.29	4.24	geranyl butyrate

[a] Temperature: 150°C; helium flow rate: 90 ml/min.

Examination of the terpenoid fraction, using a column coated with LAC-4-R777, (Lipsky and Landowne, 1959) gave chromatograms showing 39 distinct peaks or zones. Of these, 35 are numbered for identification. Again there were near the origin a few small peaks (four) that present no consistent pattern from sample to sample as to retention distance and amount. Peak identities were assigned as discussed previously. The data are presented in the form of relative corrected retention volumes (Table 6). The peaks that exceeded the 2% limit of agreement were: peak 6, isobutyl acetate (5.1% difference); peak 12, *n*-octanal (2.6% difference); peak 13, 3-hexen-1-ol (2.8% difference); peak 16, 3-hepten-1-ol (2.1% difference); peak 17, *n*-octyl acetate (2.1% difference); peak 20, linalyl acetate (4.2% difference); peak 25, terpinyl acetate (2.2% difference); peak 26, citronellol (2.4% difference); and peak 30, D-carvone (3.3% difference). Again, these differences are within the 7% limit of agreement found by Bernhard (1957) and James (1956).

Correlation between both sets of relative corrected retention volume data obtained with two stationary liquid phases, LAC-2-R446 and LAC-4-R777, was good for the following compounds: D-limonene, *n*-octanal, *n*-nonanal, 3-hepten-1-ol, *n*-octyl acetate, *n*-decanal, linalool, *n*-undecanal, bornyl acetate, isopulegol, *n*-decyl acetate, *n*-dodecanal, borneol, terpinyl acetate, citronellol, neral, geranyl acetate, geraniol, citral, D-carvone, and *trans*-carveol.

As early as 1900 Stephan reported the presence of *a*-terpinene (?) and caprylic acid in oils from the sweet orange. An extensive investigation by Hall and Wilson (1925) of the volatile constituents of Valencia orange juice revealed ethanol, acetone, acetalde-

Table 6. Relative corrected retention volumes of the components of the terpenoid fraction of a cold-pressed California Valencia orange oil. Stationary phase: LAC-4-R777.[a] (n-decanal = 1.00).

	V_R^o/V_R^c		
Peak	Unknown	Known	Compound
1	0.056	......	....
2	0.077	......	....
3	0.113	......	....
4	0.123	......	....
5	0.138	0.136	α-pinene
6	0.164	(0.158)	(isobutyl acetate)
7	0.185	......	....
8	0.215	0.217	β-pinene
9	0.236	0.238	n-hexanal
10	0.292	0.294	D-limonene
11	0.405	......	....
12	0.482	(0.495)	(n-octanal)
13	0.559	(0.575)	(3-hexen-1-ol)
14	0.626	......	....
15	0.687	0.701	n-nonanal
16	0.774	(0.791)	(3-hepten-1-ol)
17	0.831	(0.849)	(n-octyl acetate)
18	1.00	1.00	n-decanal
19	1.14	1.15	linalool
20	1.23	(1.18)	(linalyl acetate)
21	1.39	1.41	n-undecanal; linalyl propionate
22	1.57	1.57	bornyl acetate
		1.59	isopulegol
23	1.74	1.71	n-decyl acetate
24	1.99	2.01	n-dodecanal
25	2.26	(2.31)	(terpinyl acetate)
26	2.40	2.36	borneol
		2.41	geraniol; α-terpineol
		(2.46)	(citronellol)
27	2.69	2.74	geranyl acetate
28	2.87	......	....
29	3.19	3.14	neral
		3.22	citral
30	3.56	(3.68)	(D-carvone)
31	3.99	4.01	*trans*-carveol
32	4.21	......	....
33	4.48	......	....
34	6.83	......	....
35	7.86	......	....

[a] Temperature: 150°C; helium flow rate: 90 ml/min.

hyde, formic acid, an amyl alcohol (probably isoamyl), an olefinic alcohol ($C_{10}H_{18}O$), phenylethyl alcohol, esters of formic, acetic, and caprylic acids, and indications of geraniol and terpineol.

Poore's (1932) monumental investigations of the chemical composition of California citrus oils was the first major work of its kind. He reported that identified substances were practically the same in commercial California Washington navel and Valencia orange oils. Besides D-limonene, which composed 90% or more of the oil, he found the following constituents: decyl aldehyde; formic, acetic, capric, and caprylic acids; octyl alcohol; an olefin alcohol, $C_{10}H_{18}O$, closely related to but not linalool; and an unidentified white amorphous compound melting at 62–63°. He also indicated that the oils contained a trace of linalool.

Naves (1932) isolated and identified D-linalool and D-α-terpineol in the oil obtained from French Guinea oranges. He later identified myrcene, limonene, terpinolene (?), "a terpene," an aliphatic terpene (probably ocimene?), octyl aldehyde (?), nonyl aldehyde, decyl aldehyde, citral, nonyl alcohol, decyl alcohol, nerol, geraniol (?), farnesol (or nerolidol), methyl anthranilate (?), and sesquiterpenes. In 1947 Naves reported finding n-dodecanal, n-2-decen-1-al, and n-2-dodecen-1-al in the essential oil of sweet oranges.

Nelson and Mottern (1934), investigating aldehydes in Florida Valencia orange oils, discovered that the sodium sulfite method of Tiemann (1898) was not effective in the recovery of citral from citrus oils. With sodium bisulfite, citral may go into solution as the stable dihydrosulfonic acid compound, from which it cannot be regenerated. That is probably why Poore (1932) was unable to identify citral in California orange oils. Later, Guenther and Grimm (1938) found that citral exists in both the α- and β- forms (geranial, the *cis*-isomer, and neral, the *trans*-isomer) in California orange oils.

Biale and Weiss (1939) reported the presence of acetaldehyde in the steam distillate of the peel of citrus fruits (non-commercial oils). Foote and Gelpi (1943) noted the presence of nonyl alcohol in Florida sweet-orange oils. Still more recently Benezet and Igolen (1946) reported the presence of *n*-octyl aldehyde, *n*-nonyl aldehyde, *n*-decyl aldehyde, and citral in European sweet-orange oils.

The presence of limonene, decyl aldehyde, citral, octyl alcohol, an olefinic alcohol, $C_{10}H_{18}O$, formic, acetic, caprylic, and caproic acids was reported in California orange oils by Schweisheimer (1955).

In the 1950's Kirchner and Miller (1957) made a massive investigation of the volatile constituents of Valencia orange juice. During the work, they distilled more than 7,000 gallons of various orange-juice samples and isolated over 36 compounds from the oil fractions. Their findings included: limonene, α-thujene (?), ethyl isovalerate, methyl α-ethyl-*n*-caproate, citronellyl acetate, terpinyl acetate, hexanal, octanal, decanal, 2-dodecenal (?), citronellal, carvone, linalool, carveol, 1-decanol, 1-octanol, α-terpineol, 1-hexanol, 3-hexen-1-ol, and some polyoxygenated compounds.

Table 7 presents a convenient summary of compounds previously reported in the volatile oils of sweet oranges. The variety reported is great, not corresponding in all regards with the present findings. In the terpene fraction, besides large amounts of limonene and myrcene, I found: α-pinene, α-phellandrene, γ-terpinene, and *p*-cymene, none of them previously reported. I was unable to identify with certainty: ocimene, terpinolene, carene, thujene, and the sesquiterpene cadinene (Kirchner and Miller, 1957).

General agreement, however, is good with findings in the literature and findings in this work on the terpenoid (oxygenated) compounds. Besides compounds previously reported, I detected: 3-hepten-1-ol, *n*-octyl acetate, *n*-undecanal, bornyl acetate, isopulegol, *n*-decyl acetate, borneol, citronellol, neral, and geranyl acetate.

One of the important differences between the current and past investigations is the use of GLC. This method affords higher resolution and permits separation under milder conditions. In addition, none of the oils examined was subjected to distillation and concomitant damage by heat. The preseparation was accomplished under the mildest possible conditions, employing room-temperature chromatographic techniques. The combination of the two methods yielded a more definitive picture of the components and decreased the number of artifacts introduced during handling and examination.

Table 7. Volatile constituents previously reported in sweet-orange oil.

Hydrocarbons	Aldehydes
limonene	citral
myrcene	*n*-decanal
ocimene (?)	*n*-nonanal
terpinolene (?)	*n*-octanal
α-terpinene	2-decenal
carene	2-dodecenal
cadinene	citronellal
α-thujene (?)	*n*-hexanal
Alcohols	Ketones
linalool	carvone
1-octanol	Esters
1-nonanol	methyl anthranilate
D-terpineol	methyl *N*-methyl-anthranilate
1-terpineol	nonyl caprylate
nerol	ethyl isovalerate
1-decanol (?)	methyl α-ethyl-*n*-caproate
geraniol (?)	citronellyl acetate
farnesol (or nerolidol)	terpinyl acetate
2-phenylethanol	Acid portion of esters
carveol	formic acid
1-hexanol	acetic acid
3-hexen-1-ol	capric acid
	caprylic acid
	butyric acid

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Effect of Pre-irradiation Heating Temperatures, Irradiation Level, and Storage Time at 34°F on the Free Amino Acid Composition of Beef[a,b]

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SUMMARY

Slices of fresh beef were heated to 130, 150, and 195°F, then irradiated at 0.1 and 5.0 megarads, and stored at 34°F for periods up to 60 days. Unheated and unirradiated beef was subjected to the same variables. No free lysine, cystine, or tryptophan was found. Heating to 150°F inhibited the release of amino acids from the parent protein. Histidine and tyrosine were observed in quantitative amounts, but only traces of alanine, glycine, leucine, threonine, and valine were observed at 60 days. Heating to 150°F completely inhibited the appearance of aspartic acid, glutamic acid, serine, methionine, and phenylalanine. Methionine and valine were affected by radiation at both levels. Serine, phenylalanine, histidine, glycine, glutamic acid, aspartic acid, and alanine were affected only at the 5.0-megarad level. Storage generally resulted in increasing quantities of the free amino acids except for arginine and proline.

Enzymatic breakdown of the protein in fresh meats begins during storage. The rate of proteolysis may be altered by, among other things, heat denaturation of the protein, irradiation, and storage temperature. The changes occurring in amino nitrogen, total soluble nitrogen, and trichloroacetic-acid-soluble nitrogen in fresh and irradiated meat stored at 34°F have been reported (Bautista *et al.*, 1961). The increase was greatest in the TCA-soluble fraction, followed by the total soluble and amino nitrogen fractions. Between 30 and 45 days of storage the rate of increase of the total soluble nitrogen fraction markedly increased in the untreated beef. This was undoubtedly due to the formation of large peptides. Irradiation at the 5.0-megarad level of fresh beef promoted the formation of peptides, for the marked increase in the total soluble nitrogen fraction occurred between 15 and 30 days of storage.

The changes that occurred may be accounted for, in part, by the release of certain amino acids from the parent protein. Zender *et al.* (1958) found that the free amino acid level of stored lamb and rabbit muscle rose during aseptic storage, and that the glycine-soluble protein level decreased. Electrophoretic examination of the protein showed new sub-unit peaks in the pattern when rabbit muscle was allowed to age 15 days at 38°C. These sub-units represented a breakdown of the protein to peptides and would be accounted for in the total soluble nitrogen fraction.

With respect to the free amino acids, Colombo and Gewasini (1956) found the distribution of free amino acid content of fresh meat to be 0.676, 0.900, 1.964, and 0.261

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mg/g of alanine, glutamic acid, cystine and leucine, respectively. When the meat was aged 12 days these amino acids were respectively 0.780, 0.964, 2.100, and 0.335 mg/g. Niewiarowicz (1958) aged both beef and pork for 18 days at 4°C. Free amino acids were detected chromatographically on the first day of storage. The notable exception was tryptophan, which did not appear until the end of 15 days of storage. The other free amino acids increased in quantity as storage time increased.

This paper reports the pattern of release from native beef protein, of the following amino acids: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, leucine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine. Also considered are the effects of heating the beef before storage and of irradiation levels of 0.1 and 5.0 megarad.

EXPERIMENTAL

Preparation of the beef and heating and irradiation treatments were described in a previous paper (Bautista *et al.*, 1961). Samples of beef were heated, irradiated, and stored at 34°F. Analyses were made immediately after irradiation and after 15, 30, 45, and 60 days of storage.

Extraction of free amino acids. The free amino acids were extracted from the beef by the method suggested by Block *et al.* (1955). Ten grams of meat were blended with absolute ethyl alcohol so that final concentration of the alcohol was 80% by volume. The insoluble material was removed by filtration and washed with 80% ethanol. Three volumes of chloroform per volume of extract were then added. After thorough mixing the preparation was allowed to stand until separated. The resulting aqueous layer was then removed and made to volume of 10 ml with 80% ethanol and stored at room temperature pending chromatography.

Determination of free amino acids. The free amino acids were analyzed quantitatively with one-dimensional paper chromatography. The amino acids were analyzed in 5 groups, depending on the solvent system used. The solvent systems for the amino acids in Groups I through IV were those suggested by Hackman and Lazarus (1956): the system for Group V was that suggested by Subramanian and Lakshminarayan Rao (1955).

The amino acids were: Group I, alanine, aspartic acid, glutamic acid, glycine, serine, and threonine; Group II, leucine, methionine, phenylalanine, valine, and tryptophan; Group III, arginine and lysine; Group IV, histidine and proline; and Group V, cystine and tyrosine. The solvent systems were: Group I, phenol (74% w/w) in a buffer of pH 10.0 (0.053*M* boric acid and potassium chloride, 0.047*M* sodium hydroxide); Group II, *n*-butanol (77% v/v), acetic acid (6% v/v), and water (17% v/v); Group III, acetone (60% v/v) in a buffer of pH 7.0 (0.040*M* dibasic sodium phosphate and 0.027*M* monobasic potassium phosphate); Group IV, aqueous acetone (60% v/v); and Group V, 7 ml of pH 1.0 buffer (50 ml of 0.2*M* potassium chloride plus 97 ml of 0.2*M* hydrochloric acid) added to 50 ml of distilled phenol. When a buffer was used in a solvent system the paper was also buffered with the same buffer.

Two Pyrex baking dishes, 10 x 15 in., were ground so that, when fitted together, an air-tight chamber would result. Twelve such units served as chromatographic chambers. The chambers were held in place in wooden racks, 4 such chambers to the rack, and each rack provided with a tilting mechanism.

Sheets of Whatman No. 1 filter paper, 8½ x 13 in., were used as the chromatograms. The paper was held in the chambers by glass rods so positioned that only one end of the paper dipped into the solvent. The tilting mechanism allowed for equilibration of the paper before dipping if necessary for a particular solvent system. The chambers were kept in a room at 24°C. Twenty-five milliliters of the specific solvent were used in ascending chromatography.

Micropipettes were used to space 8 spots on one paper. A 20-μl spot was found to be adequate for all free amino acids extracted from beef held at 34°F. Chromatograms developed with the Group I solvent system required 24-hour equilibration before being dipped into the solvent.

Chromatograms were developed by spraying with the following solvents: Group I, 2% ninhydrin in ethanol with 2% acetic acid; Group II, 1% ninhydrin in ethanol with 0.25% triethylamine, and for tryptophan, 1 g of *p*-dimethylaminobenzaldehyde in 90 ml of acetone with 10 ml of concentrated hydrochloric acid; Group III, 2% ninhydrin in ethanol; Group IV, for histidine, diazotized sulphanilamide solutions, and for proline, 1% isatin in ethanol; Group V, 0.4% ninhydrin in ethanol containing 4% acetic acid.

All chromatograms were air-dried. The amino acid content was determined by the density of its spot at the time of greatest density, using a Photovolt densitometer, Model 525, with a No. 47 Wratten Filter.

Calculations were carried out by the method of Block *et al.* (1955). All free amino acids were calculated to mg per 100 g of dry meat, using ratio and proportion methods. The reading for the maximum density of a sample spot was compared with

the reading for known standards developed on the same paper.

RESULTS AND DISCUSSION

In general, leucine, valine, alanine, glutamic acid, proline, and tyrosine were quantitated in fresh, untreated beef, and the level of these amino acids increased at each period of storage (Fig. 1). The amount of free arginine was approximately the same initially as that after 60 days at 34°F. In fresh, untreated beef, either no free amino acid or trace amounts were initially found for the other amino acids. Histidine appeared at 30 days of storage, serine, glycine, threonine, and phenylalanine at 45 days, and aspartic acid and methionine at 60 days.

Tyrosine, leucine, proline, threonine, and arginine were not affected by irradiation at either of the two levels used. Serine, phenylalanine, methionine, histidine, glycine, glutamic acid, aspartic acid, and alanine showed a marked increase when the raw beef was subjected to 5.0 megarad but not at 0.1 megarad. Methionine and valine showed a marked increase following irradiation at either 0.1 or 5.0 megarad. Methionine was initially found at 60, 45, and 30 days, respectively, in raw unirradiated beef, 0.1 megarad, and 5.0 megarads. The amount of free valine in beef was about the same, irrespective of the two irradiation levels used.

Heating beef to 130°F, followed by immediate cooling, delayed the appearance of free tyrosine for 30 days; leucine, alanine, glutamic acid, and histidine for 45 days; and valine and glycine for 60 days. Only trace amounts of methionine, phenylalanine, threonine, serine, and aspartic acid were found in similarly treated beef held 60 days at 34°F.

Heating to 150°F further delayed the appearance of the amino acids. Quantitative amounts of histidine and tyrosine were found after 45 days of storage. Trace amounts of alanine, glycine, leucine, threo-

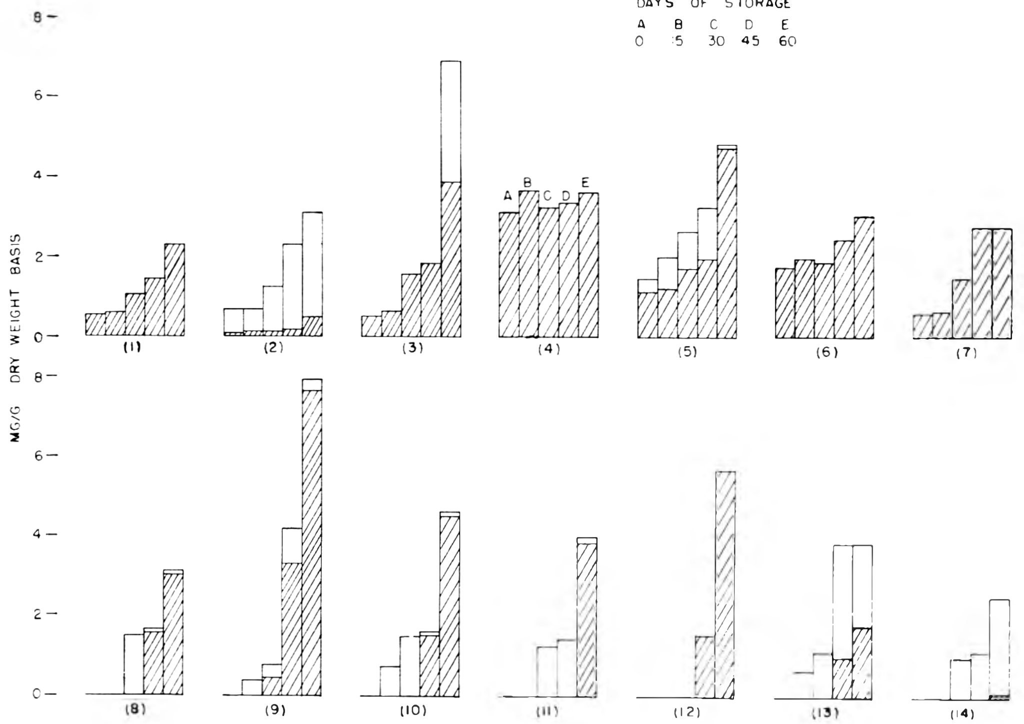

Fig. 1. Free amino acids of fresh beef stored 60 days at 34°F. (1) leucine, (2) valine, (3) alanine, (4) arginine, (5) glutamic acid, (6) proline, (7) tyrosine, (8) serine, (9) histidine, (10) glycine, (11) aspartic acid, (12) threonine, (13) phenylalanine, and (14) methionine. [hatched box] unirradiated beef; [open box] beef irradiated to 5.0 megarads.

nine, and valine were observed at 60 days. No aspartic or glutamic acid, serine, methionine, or phenylalanine were found at 60 days.

Heating to 195°F effectively inhibited the appearance of free alanine, aspartic acid, glutamic acid, glycine, serine, leucine, methionine, phenylalanine, threonine, and valine. Tyrosine and histidine were found at 45 days in unirradiated beef heated to 195°F. The level of tyrosine and histidine increased with an additional 15 days of storage.

Free arginine and proline were found in quantitative amounts at all periods of storage, irrespective of heat treatment or irradiation dose. The amount of free arginine in unirradiated raw beef was 3.15 mg/g initially, and 3.64 mg/g after 60 days (Fig. 1). Heating to 130°, 150°, and 195°F respectively reduced initial values to 2.04, 1.29, and 0.59 mg/g. Heating thus reduced initial values, but storage did not result in an increase of the initial quantity of free arginine. Heating unirradiated beef resulted in a progressive reduction in the initial value for free proline. As with arginine, the amounts were not increased in storage.

No free lysine, tryptophan, or cystine was found in beef samples stored 60 days at 34°F.

Microbial changes. In the unirradiated unheated samples, microbial growth became apparent in the 30–45-day period. This was offset by increasing the pre-heating temperature. The effect of microbial contamination in the 0.1-megarad samples was not apparent until the 45–60-day period, and then only in the unheated beef. No evidence of microbial growth could be demonstrated in the beef irradiated at 5.0 megarad.

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Enzyme-Catalyzed Breakdown of Dehydroascorbic Acid in Plant Tissue

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Homogenates of plant material were examined for enzymatic delactonization of dehydroascorbic acid and oxidation of diketogulonic acid. Bitter gourd fruit, cauliflower, and the leaves of the tapioca plant contain an enzyme that brings about conversion of dehydroascorbic acid to diketogulonic acid. Tapioca leaves are able to effect, in addition, a significant enzyme-catalyzed degradation of diketogulonic acid. The leaves of the tapioca plant contain some material that stabilizes ascorbic acid against oxidation to dehydroascorbic acid during homogenization in a Waring blender.

Progress has been considerable in elucidating the pathways of biosynthesis of ascorbic acid (Brown, 1960) but information on its catabolic pathway(s) is meager and confined mostly to whole animals and animal tissue (Lamden and Chrystowski, 1954; Hellman and Burns, 1955, 1958; Burns *et al.*, 1951, 1954, 1956, 1958; Curtin and King, 1955; Chan *et al.*, 1958; Kanfer *et al.*, 1960). It is generally agreed that dehydroascorbic acid (DHA) and diketogulonic acid (DKA) are formed as intermediates before further oxidation of reduced ascorbic acid (AA) can take place. The conversion of AA to DHA can be catalyzed both enzymically (specific as well as nonspecific) and nonenzymically (Harris, 1954a). As for the transformation of DHA to DKA, the nonenzymic reaction was demonstrated *in vitro* by Herbert *et al.* (1933), Borsook *et al.* (1937), Penney and Zilva (1943), and Mapson and Ingram (1951). Mills *et al.* (1949) observed that there was about 50% conversion of DHA into DKA in potato homogenates on storage in the cold for 6 hr, but they did not seem to have envisaged a possible enzymatic route in addition to the chemical transformation. According to Harris (1954b), no definite evidence is available for any enzymically catalyzed transformation of DHA to DKA. In the absence of definite experimental evidence, the pathway of the biological degradation of DKA has been considered to follow the chemical pathway closely. Vinokurova and Kuznetsova (1948) found that DKA was enzymically cleaved on incubation with rabbit-liver slices. Working with homogenates, particulate fractions, and supernatants of rat and guinea-pig liver, De Salegni *et al.* (1954) demonstrated that soluble enzyme fractions brought about the transformation of DHA beyond the DKA stage. The products were not identified, the postulated degradation being based solely on reaction with 2,4-dinitrophenylhydrazine (DNPH). Burns *et al.* (1958) showed that the soluble fraction of rat-kidney homogenates contained an enzyme system carrying the catabolism of DHA beyond DKA, and that CO_2 was one of the products formed. The decarboxylating enzyme from rat kidney was partially purified by Kanfer *et al.* (1960), who identified L-lyxonic and L-xylonic acids as the degradation products arising from DKA. A survey by the authors showed that the enzyme was not restricted to rat kidney, but also occurred in the kidneys of guinea pig, hog, and calf, as well as in the livers of both rats and guinea pigs.

Whereas some progress has been made on elucidation of the enzymatic pathway of the catabolism of DHA in animal tissue, no information seems available on its possible enzymatic breakdown in higher plants. The present investigation had a twofold aim: to establish whether plant tissues possess an enzymic mechanism for delactonization of

DHA to DKA, and whether they contain an enzyme system for the breakdown of DKA. During these investigations the plant tissues were also examined for any protector against AA destruction. A preliminary note has covered one aspect of the problem (Tewari and Krishnan, 1960).

MATERIALS AND METHODS

Preparation of homogenates. Homogenates were prepared by grinding 10-g representative lots of fresh plant tissue with about 40 ml of ice-cold acetate buffer, 0.2*M*, pH 5.0, for 3 min in a Waring blender at full speed. The micro-monel-metal and glass bowl of the blender were kept externally cooled by packing with ice in order to minimize rise of temperature. The slurry was squeezed through muslin, the debris on the cloth rejected, and the filtered suspension made to volume to give a 20% homogenate.

Enzyme assay. Ten ml of the homogenate was mixed with 17 ml of acetate buffer, pH 5.0, and 3 ml of DHA adjusted to pH 5. Five-ml portions were taken, immediately on mixing with the substrate and again after 60 min at 37°. These were run into 20 ml of 5% HPO_3 containing 0.5 g of $SnCl_2$ to arrest the reaction; the suspension was made to 100 ml with 5% HPO_3 and filtered. Aliquots of the filtrate were used in estimating DKA, DHA, and AA. Delactonase activity was expressed in terms of percentage of DHA disappearing from the system (after correcting for any AA formed) and also the amount of DKA formed, calculated as a percentage of initial DHA. The amount of DKA disappearing from the system gave an estimate of the degradation of the carbon chain beyond DKA. A control experiment was run simultaneously, wherein a heat-treated homogenate (20 min in boiling water and readjustment to initial volume) was used in place of the fresh homogenate. DHA used in the assays was prepared by the oxidation of AA dissolved in glass-distilled water (1 mg/ml) with liquid bromine (Salegni *et al.*, 1954).

Assay of DKA, DHA, and AA. These assays were carried out by the DNPH method as described by the Association of Vitamin Chemists (1951), with minor modifications. It may be pointed out that the treatment with H_2S had to be continued for more than 15 min with some extracts because of a delay in the separation of SnS. The total gassing period, however, was kept constant for the experimental and control samples and for the zero-min and 60-min aliquots. It may also be mentioned that the addition of H_2SO_4 sometimes yielded a pale-yellow color in the blank tubes employed in color matching, but the intensity was not strong enough to affect the color comparisons adversely. A Klett-Summerson colorimeter, with filter No. 50, was used for measuring the intensity of the colors. The values for DKA, DHA + DKA, and AA + DHA + DKA (total ascorbic acid) are expressed in equivalents of μg of AA.

RESULTS

In all, 7 samples of plant tissue were examined in the form of whole homogenates for enzyme systems involved in the hydrolysis of DHA and eventual breakdown. The data (Table 1) reveal the following:

Enzymic delactonization of DHA. Tapioca leaves, bitter gourd fruit, and cauliflower possessed an enzymic mechanism for converting DHA to DKA, since the proportion of DHA disappearing (after correcting for AA formation in tapioca leaves) was significantly higher in the experimental tubes than in the heat-treated controls. The proportion of DKA formed was also higher in the experimental systems derived from bitter gourd and cauliflower than in their respective controls, but such a direct calculation was not possible with tapioca leaves, since the amount of DKA was less at the end of incubation than at the beginning.

Enzymatic degradation of DKA in tapioca-leaf homogenates. The assays with tapioca-leaf homogenate were marked by the fact that, unlike in the experiments with the other plant samples, there was a significant diminution in DKA at the end of incubation in the experimental system. The heat-treated controls were normal in that there was a slight increase in DKA on incubation. The proportion of DHA disappearing from the system was considerable (56–62%), but about half of it was apparently converted to AA, the rest changing to DKA. The amount of DKA undergoing enzymic destruction was given by the disappearance of total ascorbic acid during incubation after correction for the loss in the blank experiment. The loss in DKA can be arrived at also from the disappearance in DHA after correction for formation of AA and the residual amount of DKA left in the system. Bearing in mind that there was already a certain amount of DKA at zero min and that this was augmented by transformation from DHA during the experiment, the net loss in DKA works out to 45–68% of initial DHA in the experimental samples, and 7–15% in the heat-treated controls. These figures clearly indicate a marked enzymic destruction of DKA in tapioca-leaf homogenates.

Nonenzymic delactonization of DHA. All the tissues examined, including tapioca leaves, effected a nonenzymic transformation of DHA to DKA, as indicated by the fact that the heat-treated homogenates caused the disappearance of 3–14%

Table 1. Alteration in the distribution of ascorbic acid, dehydroascorbic acid, and diketogulonic acid—fresh homogenates of plant tissues with dehydroascorbic acid incubated 60 min at 37° and pH 5.0, compared with control homogenates (heat-treated 20 min in boiling water before incubation under the same conditions).

	μg/5 ml												DHA Loss (%)		DKA formed (% of initial DHA)	
	DKA				DKA + DHA				Total							
	Fresh		Control		Fresh		Control		Fresh		Control					
	Begin	End	Begin	End	Begin	End	Begin	End	Begin	End	Begin	End	Fresh	Control	Fresh	Control
Cauliflower (*Brassica oleracea botrytis*)	257	317	223	240	758	744	497	487	696	703	459	445	14.8	9.9	12.0	6.2
added Mn^{++} to 0.001*M*	216	273	209	227	676	673	524	524	641	630	492	496	13.0	5.7	12.4	5.7
Bitter gourd (*Momordica charantia*) fruit	321	424	224	248	897	896	476	479	690	786	393	428	17.9	8.3	17.9	9.5
	361	414	243	250	957	950	482	482	729	772	400	414	10.1	2.9	8.9	2.9
Tapioca (*Manihot utilissima*) leaf	271	239	271	286	461	322	464	450	914	829	850	836	56.3	15.0	?	7.8
	240	171	236	244	484	262	480	444	1018	807	960	924	62.7	18.0	?	3.3
Bougainvillea (*Bougainvillea spectabilis*) leaf	297	324	255	267	617	613	495	488	514	522	411	427	9.7	7.9	8.4	5.0
Amla (*Phyllanthus emblica*) leaf	246	294	187	209	705	705	462	455	638	668	554	514	10.5	10.5	10.5	8.0
Drum stick (*Moringa pterigosperma*) fruit	589	669	193	218	1491	1473	466	458	1363	1346	458	458	10.9	12.1	8.9	9.2
	714	736	254	264	1500	1500	482	479	1357	1357	493	479	2.8	5.7	2.8	4.4
leaf	322	437	185	222	1022	1037	444	444	800	844	382	382	14.3	14.3	16.4	14.4
	422	472	250	268	943	943	439	439	872	857	418	425	9.6	9.5	9.6	9.5

DHA, which could be accounted for as DKA formed at the end of the incubation period.

Nonenzymic degradation of DKA. The disappearance of DHA in the experimental samples on incubation is due to transformation into DKA and, as with tapioca leaves, to AA. In the heat-treated samples the disappearance of DHA is due solely to DKA formation by chemical mechanism, since enzymic formation of AA is ruled out. Therefore, the difference between the percentage of DHA disappearing and DKA formed therefrom in the heat-treated homogenates should be a measure of the amount of DKA undergoing chemical degradation. This loss is given also by the decrease in total ascorbic acid on incubation. All tissues examined, except drum-stick leaves and bitter gourd, seemed to catalyze a certain measure of nonenzymic decomposition of DKA.

Discrepancy in the analyses of DHA + DKA and AA + DHA + DKA (total ascorbic acid). The fact that the value for total ascorbic acid was not higher than the value for DHA + DKA indicated that the very process of homogenization resulted in complete transformation of AA to DHA and DKA in all the samples examined except tapioca leaves, in which case the vitamin was present almost entirely in reduced form. It will be noticed that the values for total ascorbic acid in these samples are actually *lower* than the values for DHA + DKA. This discrepancy was reported by Tewari and Krishnan (Tewari and Krishnan, 1961), who ascribed it to losses during H_2S treatment of samples containing a preponderance of DHA and DKA. The heat-treated homogenates of Amla leaves were abnormal, since the values for total ascorbic acid were higher than for DHA + DKA, even though the reduced form had been completely transformed into these two products in the fresh homogenates, which gave a value for total ascorbic acid lower than that for DHA + DKA.

Destruction of DHA and DKA by heat-treatment. When these homogenates were heated 20 min in boiling water destruction of DHA and DKA was almost complete, so that the zero-min values for DHA + DKA in the heat-treated controls corresponded to the vitamin subsequently added in the form of DHA, that is, about 400–500 μg. It is desired to draw attention to the fact that the zero-min analyses of the controls indicate that DKA constitutes about 40–50% of total vitamin added in the form of DHA. Tewari and Krishnan (1961) pointed out that the samples of DHA prepared by oxidation of AA with liquid bromine always contained a certain proportion of DKA. The content of DKA in the samples in the present investigation is definitely higher than those reported earlier. This may be ascribed to the fact that the pH of the solution was raised to 5.0, and several minutes lapsed before addition of the substrate to the assay system. It will be obvious that the DKA in the experimental samples is derived from any formed from tissue AA during homogenization plus that present in the DHA added as substrate. In tapioca-leaf homogenates, which contained the vitamin almost entirely as AA, there was very little destruction in heating.

DISCUSSION

In view of the known occurrence of lactonases in animal tissue (Winkelman and Lehninger, 1958; Dowben, 1959; Yamada *et al.*, 1959; Brodie and Lipmann, 1955) and microorganisms (Brodie and Lipmann, 1955; Jermyn, 1960; Sistrom and Stanier, 1954), it may be anticipated that plant tissue may contain a similar enzyme effecting the conversion of DHA to DKA. The search for this enzyme is complicated by the fact that spontaneous transformation of DHA is very considerable at pH values near neutrality, and may mask the enzymatic reaction. We chose a distinctly acid pH (5.0) in the assay system in order to minimize chemical transformation. However, this may not be the optimum pH for the enzymic action. Another difficulty was the method of assay of AA, DHA, and DKA. As far as we are aware, the only method for simultaneous estimation of the three forms is based on the reaction with DNPH. This method of assay, adopted in this investigation, is very laborious and demands a high order of skill. An unexpected source of error was encountered (Tewari and Krishnan, 1961) when samples containing ascorbic acid mostly in the form of DHA and DKA were found to have a value for total ascorbic acid less than for DHA + DKA. The values for DHA + DKA are obtained by direct coupling with DNPH, and are likely to be correct. DKA and total ascorbic acid, in contrast, are assayed after H_2S treatment, and their values are likely to be affected by errors inherent in the H_2S treatment. AA is obtained as the difference between total and DHA + DKA, and DHA as the difference between DHA + DKA and DKA; it is, therefore, apparent that all the three individual values might be subject to some error. Since the experimental sam-

ples and the controls were assayed under identical conditions, the general conclusions on transformation of DHA and DKA may be considered valid.

The results show that bitter gourd fruit, cauliflower, and tapioca leaves contain an enzyme capable of effecting delactonization of DHA to DKA. On incubation with fresh homogenates, DHA is transformed into a product that gives the osazone with DNPH but cannot be reduced back to AA with H_2S, which two facts point to DKA as the reaction product. It is not clear whether the enzyme is capable of effecting a reversal of the reaction—lactonization of DKA to DHA.

Experiments with tapioca leaves indicate that they contain, in addition, an enzyme system that effects scission of the DKA molecule. Here again, the conclusion is based on the reaction with DNPH, not on actual identification of the products of reaction. DHA and DKA were transformed into products that could no longer react with DNPH, either directly or after bromine oxidation, thereby leading to the conclusion that the DKA molecule underwent rupture.

There have been reports on the occurrence in plants of stabilizers against oxidative breakdown of AA. Somogyi (1944) reported that certain fruits and vegetables contain a substance that effectively inhibits both the enzymic- and Cu^{++}-catalyzed oxidation of AA. The substance was not identified, however, nor was the mechanism of the protective action elucidated. Damodaran and Nair (1936) observed that AA present in Indian gooseberry (Amla) juice was remarkably stable to storage, and correlated the stability to the occurrence of a tannin. Since added Cu^{++} overcame the natural inhibition, it was concluded that the tannin functioned by complexing with metal ions. Ratnam and Srinivasan (1959) found that the tannin also protected AA of gooseberry against loss in heat treatment. Kardo-Sysoeva and Nisenbaum (1938) and Giri and Krishnamurthy (1940) also reported substances in plant tissue protecting AA against destruction. Jackson and Wood (1959) recently isolated from rose hips compounds that stabilized AA against specific and nonspecific enzymic oxidation. The present results show that, among all the plant tissues examined, tapioca leaves are an exception in that the fresh homogenates prepared with the Waring blender contain the vitamin in essentially the reduced form, whereas it is present entirely in the form of DHA and DKA in the homogenates of other plant material. Mills *et al.* (1949) reported that potato slurries prepared in a Waring blender contained all the vitamin in the form of DHA and, to a small extent, DKA. The inference may be drawn that tapioca leaves contain some material that helps maintain the vitamin in the reduced form under conditions of homogenization that lead to nonenzymic oxidation to DHA in several other plant tissues.

Fresh homogenates of the leaves of Amla resemble the homogenates of many other plant tissues in that the vitamin is present almost entirely in the form of DHA and DKA, so that assays carried out in the experimental samples gave values for total ascorbic acid that were lower than DHA + DKA. On the other hand, the corresponding values for the heat-treated homogenates were abnormal, since DHA + DKA was significantly lower than total ascorbic acid. It may be inferred that, as a result of heat-treatment of homogenate, AA was formed and, being comparatively stable, survived heating, whereas DHA and DKA were destroyed. The existence of an "ascorbigen" (Ghosh and Guha, 1939) in Amla leaves would explain the release of AA on the heating of leaf homogenates.

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Factors Affecting the Water Retention of Beef. V. Variation of the Zinc-Containing Enzymes

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SUMMARY

A comparative study of the lactic dehydrogenase, glutamic dehydrogenase, carbonic anhydrase, zinc, water-soluble nitrogen, moisture, and protein content of six different muscles from three bovine animals was conducted in an investigation of factors contributing to differences in the ultimate pH values of the muscles. The results indicate that variation was considerable in content of zinc, lactic dehydrogenase, glutamic dehydrogenase, and carbonic anhydrase. A highly significant direct correlation was found between pH and zinc content, and a highly significant inverse correlation between lactic dehydrogenase and pH. Lactic dehydrogenase significantly correlated with soluble nitrogen content. No relation was found between glutamic dehydrogenase, carbonic anhydrase, and pH.

Previous studies have indicated marked differences in the water retention, pH value, and zinc content of different muscles of bovine animals (Swift and Berman, 1959; Swift *et al.*, 1960). A highly significant positive correlation was found between water retention, zinc content, and pH. The parallel relationship of zinc and pH, which is in contrast to that between pH and the divalent cations calcium and magnesium, suggested that zinc may participate as a component of enzymes, the action of which may in part determine pH differentials. Carbonic anhydrase, glutamic dehydrogenase, and lactic dehydrogenase, three enzymes present in muscle tissue, contain zinc as an integral part of the protein molecule (Vallee, 1956; Vallee and Wacker, 1956). Removal of the metal results in irreversible inactivation of the enzyme.

Carbonic anhydrase catalyzes the reaction $CO_2 + H_2O \leftrightharpoons H_2CO_3$. Zinc is present to the extent of 0.33% (Keilin and Mann, 1940). Studies (Ashby and Chan, 1943) of carbonic anhydrase in human autopsy tissue suggest that carbonic anhydrase plays a role in intermediary metabolism of carbohydrate as an adjunct in decarboxilating reactions. Glutamic dehydrogenase contains 4–5 zinc atoms per molecule (Vallee *et al.* 1955). The enzyme catalyzes the reaction α-ketoglutarate + DPNH + NH_4^+ → DPN^+ + H_2O + L-glutamate, a reaction that is an important connecting link between carbohydrates and proteins. Lactic dehydrogenase catalyzes the reaction pyruvic acid + DPNH $\leftrightharpoons$ DPN^+ + lactic acid, the end product of muscle glycolysis.

The studies of Andrews *et al.* (1952) and Grant (1955) have indicated the post-mortem stability of the glycolytic and of the citric acid cycle enzymes. Post-mortem inactivity was shown to be due to substrate dearth. Therefore, determination of the rate of substrate turnover of the three zinc-containing enzymes would be a measure of their activity during post-mortem metabolism, the period during which the ultimate pH is attained.

The present investigation followed the general procedure of previous papers of the series. Its purpose was to compare the enzyme and zinc content of six muscles from three animals; it involved determination of ultimate pH values, lactic dehydrogenase (LDH), carbonic anhydrase (CA), glutamic dehydrogenase (GDH), zinc, soluble nitrogen, water, and protein content. Ex-

periments were also conducted to determine the stability and optimum conditions for measurement of the three enzymes.

EXPERIMENTAL

Preparation of samples. Carcasses of three animals were obtained immediately after slaughter. Six muscles designated in Table 1 were prepared as previously described (Berman, 1960). For the enzyme determinations, 10-g portions of the meat were weighed into beakers, covered with parafilm, and stored at −30°C until used. The remaining meat was stored at 2°C for determination of pH, zinc, water-soluble nitrogen, moisture, and protein. At the time of storage, samples were about 5 hr post-mortem.

Table 1. Muscles selected and locations.

Muscle		Location	
No.	Name	Forequarter	Hind-quarter
1	Longissimus dorsi	Rib, chuck	
3	Semimembranosus		Round
4	Serratus ventralis (thoracic part)	Rib, chuck	
5	Rectus abdominis	Plate	Flank
6	Semitendinosus		Round
8	Trapezius	Rib, chuck	

Determining CA, LDH, and GDH. Homogenates for the determination of CA, LDH, and GDH were prepared by blending 10 g of frozen meat with 100 ml of ice-cold water for 1 min in a Serval Omnimixer immersed in an ice bath. (Mention of trade names in this paper is for identification and implies no endorsement of the products.)

CA activity was measured manometrically at 15°C by the method of Krebs and Roughton (1948). Twenty-five ml of the slurry were diluted to 100 ml with cold 0.05% Difco bacto-peptone solution. Scott and Mendive (1941) showed that stability and activity are maximum when CA solution is diluted with 0.05% peptone solution. To the main compartment of a Warburg reaction flask were added a few glass beads, 0.5 ml of the diluted slurry (0.5 ml of 0.05% peptone was used for the blank or uncatalyzed reaction), and 1 ml of a phosphate buffer solution prepared by mixing 300 ml of 0.1*M* Na_2HPO_4 with 200 ml of 0.1*M* KH_2PO_4. To the side arm of the flask was added 1 ml of freshly prepared 0.05*N* $NaHCO_3$. The flask was equilibrated for 5 min at 15°C. At zero time, after equilibration, the two solutions were mixed and shaken at 160 oscillations per min, and, as suggested by Mitchell *et al.* (1945), pressure readings X_1, X_2 · · · · X_n were taken at equal intervals of time (10 readings at 30-sec intervals) beginning 30 sec after mixing. The readings were paired for 60-sec intervals and differences taken, i.e., $(X_4 - X_2)$, $X_5 - X_3)$ · · · · $(X_{11} - X_9)$. The velocity constant was obtained by plotting the common logarithm of the differences against time. The slope of the line multiplied by 2.303 gives the velocity constant. The amount of enzyme activity was found by measuring the velocity constants for the uncatalyzed and catalyzed reactions. The activity is expresed by the unit K_D defined by the equation $K_D = K_C - K_O$ where K_C = velocity of the catalyzed reaction; K_O = velocity of the uncatalyzed reaction. Values for K_D are plotted against the logarithm of the amount of meat used in the determination, and a straight line is drawn (Altschule and Lewis, 1949). By extending this line to zero dilution, an extrapolated value for 1 g of undiluted meat is obtained.

The method of Robins *et al.* (1956) was adapted for determination of GDH activity. Twenty-five ml of the homogenate were diluted to 50 ml with 0.1*M* sodium barbital buffer, pH 8.0. One-tenth ml of the diluted slurry was transferred to a test tube immersed in an ice bath. One ml of a buffer substrate solution, prepared by mixing 18 ml of 0.05*M* sodium barbital buffer, pH 8.0, with 135 mg diphosphopyridine nucleotide (DPN) and 1.2 ml of neutralized 4.5*M* sodium glutamate, was added. For the blank determination, 1.2 ml of 0.05*M* sodium barbital, pH 8.0, were substituted for the 4.5*M* sodium glutamate. The contents of the tubes were mixed and the tubes capped with parafilm. The tubes were then placed in a water bath at 32°C for exactly 60 min. The tubes were returned to the ice bath, and 1 ml of 0.5m*M* 3-quinolylhydrazide (QH) (in 0.18*N* HCl) was added. The contents of the tubes were mixed by tapping and were then allowed to stand 1 hr at room temperature (20–25°C). The samples were then diluted with 4 ml of 0.01*N* HCl. The absorption of the samples at 350 mμ was determined 10 min after dilution. Calculations were made according to the following equation:

$$\frac{\text{O.D. with substrate} - \text{O.D. without substrate}}{\text{weight of meat in aliquot}} \times \frac{\text{final volume}}{E_t} \times 1000 = \text{moles of substrate oxidized}$$

per kilo per hour,

in which E_t = molar extinction coefficient at 305 mμ of QH − α-ketoglutaric acid, plus that of reduced diphosphopyridine nucleotide (DPNH) in the presence of QH. The E_{305} for the QH −

Table 2. Effect of blending time on enzyme activity.

Enzyme	30 sec	60 sec	120 sec	480 sec
Lactic dehydrogenase [a]	3.65	3.62	3.56	3.62
Glutamic dehydrogenase [b]	411	416	411	406
Carbonic anhydrase [c]	0.753	0.784	0.773	0.829

[a] Moles substrate oxidized per kg wet weight per 3 min.
[b] Micromoles substrate oxidized per kg wet weight per hr.
[c] Log of the pressure change (K_D) per min per g wet weight.

α-ketoglutaric acid product is 21,700, and the DPNH in the presence of QH has an E_{340} = 5960 at 10 min; therefore, E_t = 21,700 − 5960 = 27,660.

The method of Meister (1950) was adapted for determination of LDH activity. The decrease in absorbance, measured at 340 mμ, due to the oxidation of DPNH to DPN, was determined as a direct measure of the reduction of pyruvate to lactate (Colowick and Kaplan, 1955). Twenty-five ml of the homogenate were diluted two-hundred-fold with 0.1*M* PO_4 buffer, pH 7.6. Three ml of a buffer substrate solution, containing one micromole DPNH, 10 micromoles sodium pyruvate, 165 micromoles nicotinamide, and 100 micromoles of PO_4 buffer, pH 7.6, were placed in a cuvette. The reaction was started by addition of 0.1 ml of the diluted homogenate. Readings were taken for 3 min at 30-sec intervals, beginning 30 sec after introduction of the homogenate. The change in absorbance during this 3-min period, converted to micromoles of pyruvate oxidized per kg wet weight of meat, represented the enzyme activity of the sample.

Determining pH, moisture, protein, water-soluble nitrogen, and zinc. pH, moisture, protein and water-soluble nitrogen were determined as described in earlier papers (Swift and Berman, 1959; Swift *et al.*, 1960). Zinc as the zinc dithizonate in carbon tetrachloride (Sandell, 1950).

RESULTS AND DISCUSSION

In determining the enzyme activity, an important consideration was determination of optimum extraction and assay conditions. Therefore, a series of experiments were conducted to determine these parameters. Results are in Fig. 1 and Tables 2 and 3. The data show that, under the conditions chosen, all three enzyme activities are linear with respect to quantity of tissue assayed, extraction is complete in 30 sec, blending does not inactivate or denature the enzyme, and quantitative extraction of the enzymes from the tissue was achieved.

Optimum conditions for the assay of GDH and LDH were at pH 8.0 in a 0.05*N* sodium barbital buffer containing 9.5 micromoles of DPN per ml and 300 micromoles of sodium glutamate per ml, and at pH 7.6 in a solution containing 0.333 micromoles DPNH, 313 micromoles sodium pyruvate, and 33 micromoles phosphate, respectively.

Table 3. Effect of meat-to-water ratio during blending on the extraction of lactic dehydrogenase (LDH), glutamic dehydrogenase (GDH), and carbonic anhydrase (CA).

Ratio of meat to water (g)	LDH [a]	GDH [b]	CA [c]
5:100	3.25	0.947	0.793
10:100	3.21	0.942	0.719
15:100	3.21	0.970	0.847
20:100	3.36	0.915	0.756

[a] Moles substrate oxidized per kg wet weight per 3 min.
[b] Micromoles substrate oxidized per kg wet weight per hr.
[c] Log of the pressure change (K_D) per min per g wet weight.

The standard error of measurement for 12 replicates was ±2.3% for LDH, ±5.7% for GDH, and ± 14.0% for CA.

Table 4 shows the average moisture, protein, and soluble nitrogen contents of six

Table 4. Average moisture, protein, and soluble nitrogen content of six muscles from each of three animals.

Muscle		% wet weight of meat		
No.	Name	Moisture	Protein (N×6.25)	Soluble nitrogen (mg/g tissue)
1	Longissimus dorsi	74.46	21.75	7.80
2	Semimembranosus	75.11	21.80	8.37
4	Serratus ventralis	74.56	18.89	5.66
5	Rectus abdominis	76.07	20.00	6.45
6	Semitendinosus	75.67	21.41	7.52
8	Trapezius	76.27	19.99	6.16

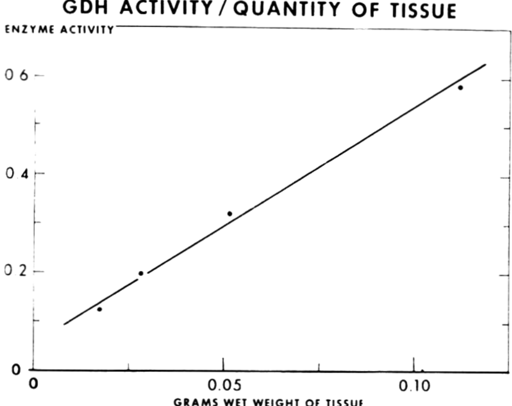

Fig. 1a. Linearity of glutamic dehydrogenase activity with respect to quantity of tissue.

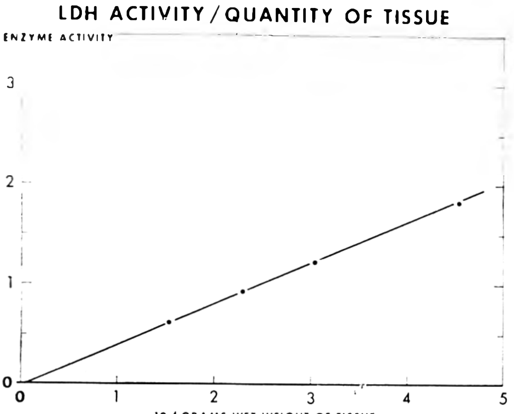

Fig. 1b. Linearity of lactic dehydrogenase activity with respect to quantity of tissue.

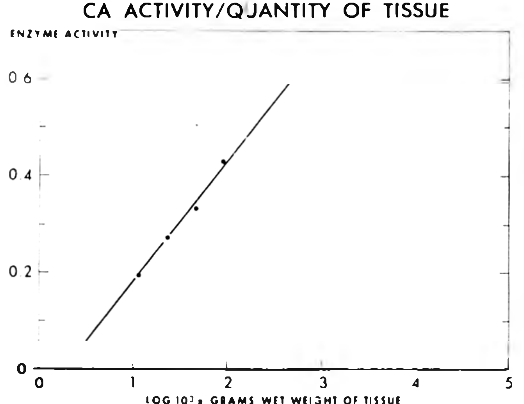

Fig. 1c. Linearity of carbonic anhydrase activity with respect to quantity of tissue.

muscles from three animals. As indicated in the data, muscles 4, 5, and 8 differ from the others in protein and soluble nitrogen content in accordance with the tendency of muscle properties to differ in patterns (Swift and Berman, 1959; Swift *et al.*, 1960).

The curve in Fig. 2 expresses the relation of LDH activity and pH. An interesting feature is that at pH 5.6 the enzyme exerts half of its optimum activity. It is significant, therefore, that even when the ultimate pH has been attained in post-rigor meat, LDH activity is capable of converting any pyruvate formed to lactic acid.

Table 5 shows the results of determination of zinc content and enzyme activity. The content of zinc and enzyme activity of individual muscles ranged widely. Muscles 4, 5, and 8 differ from the others in LDH activity. The specific activities (enzyme activity/soluble nitrogen) indicate that the enzyme is a smaller percentage of the total water-soluble fraction in muscles 4, 5, and 8 than in muscles 1, 3, and 6. Similar results were reported by Kronman and Winterbottom (1960) relative to the enzyme aldolase.

The data in Table 6 show the results of statistical analyses of the relations between pH, soluble nitrogen, and components of the muscles. The results indicate that pH was directly related to zinc content and inversely related to LDH activity. The latter supports the view that variations in the amount of lactic acid (Bate-Smith and Bendall,

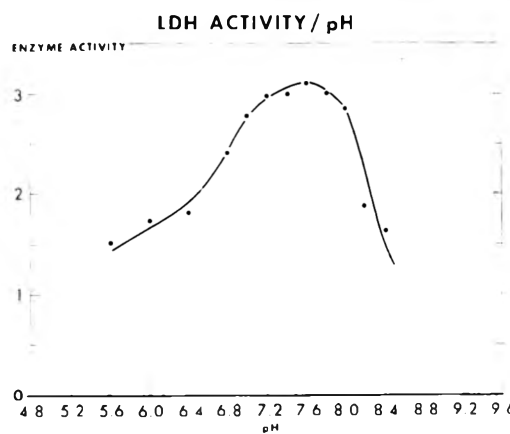

Fig. 2. Enzyme activity vs pH of reaction solution.

Table 5. Average zinc content and activity of lactic dehydrogenase (LDH), glutamic dehydrogenase (GDH), and carbonic anhydrase (CA) in six muscles from three animals.

Muscle						
No.	Name	Zn [a]	LDH [b]	GDH [c]	CA [d]	pH
1	Longissimus dorsi	4.60	3.53	660.6	0.590	5.54
3	Semimembranosus	3.79	3.73	729.9	0.632	5.51
4	Serratus ventralis	6.49	1.08	960.1	0.626	5.78
5	Rectus abdominis	5.58	2.58	674.5	0.660	5.68
6	Semitendinosus	3.60	3.74	813.1	0.468	5.51
8	Trapezius	5.03	2.14	836.2	0.473	5.65

[a] Milligrams per 100 g wet weight of tissue.
[b] Moles substrate oxidized per kg wet weight per 3 min.
[c] Micromoles substrate oxidized per kg wet weight per hr.
[d] Log of the pressure change (K_D) per min per g wet weight.

Table 6. Statistical relationships between properties of six muscles.

	Correlation coefficient	Probability [a]
Correlation with pH		
Zinc	+0.966±0.090	.01
Lactic dehydrogenase	−0.972±0.056	.01
Glutamic dehydrogenase	+0.330±0.318	n.s.
Carbonic anhydrase	−0.609±0.270	n.s.
Correlation with soluble N		
Lactic dehydrogenase	−0.931±0.123	.01
Glutamic dehydrogenase	−0.541±0.284	n.s.
Carbonic anhydrase	+0.215±0.160	n.s.

[a] Five degrees of freedom.

1956) may be responsible for ultimate pH differences in muscles.

Previous work (Swift *et al.*, 1960) has shown the marked decrease in the glycogen content of muscles attaining low ultimate pH values (Nos. 1, 3, and 6) in contrast with the lesser decrease in those attaining higher ultimate pH values (Nos. 4, 5, and 8). This was explained on the basis that ultimate pH varied with the amount of glycolysis occurring prior to ATP disappearance and that this interval was affected by variations in rates. The present results indicate that the variation in rates was associated with parallel variations in the capacity of the glycolytic enzyme systems in the muscles.

The data in Table 6 show that soluble nitrogen content was related directly to LDH activity. No relation was found between GDH and CA activity and the pH or soluble nitrogen.

The results show that, of the three zinc-containing enzymes, only LDH plays a significant role in influencing the ultimate pH of meat. The direct, highly significant correlation between pH and zinc content remains to be explained.

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The Microbicidal Activity of Gaseous Propylene Oxide and Its Application to Powdered or Flaked Foods

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SUMMARY

The sporicidal activity of gaseous propylene oxide was determined at several concentrations and at low and high relative humidity (RH). At 37°C, 85% RH, and a concentration of 1250 mg/L, propylene oxide will kill 90% of a population of dry spores of *B. subtilis* var. *niger* in one hour. If RH is lowered to 25% under the same conditions, 90% of the spores are killed in 40 minutes. Microbial decontamination of several flaked or powdered foods with propylene oxide was determined in 4-L rolling jars or in a 4-cu-ft revolving blender. The bacterial count of cereal flakes inoculated with dry spores of *B. subtilis* var. *niger* was reduced 95% in 4 hours at 37 and 55°C, but further reduction in count was achieved only after prolonged holding times. Studies with cocoa powder showed that exposure to a propylene oxide concentration of 1–2% by weight of cocoa (or 1000–2000 mg/L of space) at 37°C for 2–3 hours reduced bacterial count by 50–70% and thermophilic count by 50–90%. Destruction of molds and vegetative bacteria in cocoa or other foods was 90–99.9% under these conditions. Destruction of the microbial flora of cocoa was greater when the treated cocoa was stored with some residual propylene oxide in bottles or plastic bags. Addition of liquid propylene oxide directly to small samples of cocoa gave good reduction of the microbial flora, but also some physical destruction of the contents.

Gaseous ethylene oxide has been widely used to reduce the microbial contamination of various dry foods. Many of these applications have been based on the patents of Griffith and Hall (1938, 1940 a, b, 1942), who presented extensive data on the treatment of gums and spices in their patent claims. Other foods treated with gaseous ethylene oxide are dried fruits (Welton *et al.*, 1946), corn, wheat, barley, and potato flour (Pappas and Hall, 1952), corn starch (Pappas and Hall, 1952), dried eggs, and gelatin (Mayr and Kaemmerer, 1959, Rauscher *et al.*, 1957). Many other dried-food products have been treated with ethylene oxide, but the procedures and results have not been published.

The Food and Drug Administration, however, recently ruled that ethylene and diethylene glycols, usually found in small quantities as hydrolytic residues from ethylene oxide treatment, are objectionable in food products or in any packaging materials that come in contact with foods. The 1958 Food Additive Amendment to the Food, Drug and Cosmetic Act required that ethylene oxide be cleared by appropriate tests before it could be used in decontamination of foods. Ethylene oxide has been granted temporary clearance till March 6, 1961, for use in dried fruits, ground spices, dried mushrooms, and edible gums. These rulings have focused attention on propylene oxide as an alternate gaseous agent for reduction of microbial contamination since its principal hydrolytic product, propylene glycol, is on the safe and acceptable list of food additives. Use of propylene oxide as a fumigant for foods has been extended to March 6, 1961, by the Food and Drug Administration.

Propylene oxide has been used on dried fruits to control spoilage (McBean and Johnson, 1959; Nury *et al.*, 1960; Welton *et al.*, 1946), but there is little quantitative data on its microbicidal activity and its application to flaked or powdered foods. This paper

presents data on the sporicidal activity of propylene oxide and on the quantitative reduction of microbial contamination in some powdered and flaked foods.

MATERIALS AND METHODS

Studies with spore strips. Washed, heat-shocked spores (65°C for 30 min) of *Bacillus subtilis* var. *niger* were placed on filter-paper strips (2×0.25 in.) and air dried. The spore count on these strips ranged from 9.5×10^5 to 1.1×10^6. These strips were stored and assayed for six months prior to use. No reduction in count occurred during this time.

The spore strips were exposed to propylene oxide in 2-L vacuum flasks or 4-L Case anaerobic jars (Case Laboratories, Inc., Chicago, Ill.). No vacuum was drawn in the flasks or jars before admission of the propylene oxide, which passed in as a gas through a valve opening or side port from a warmed side-arm tube. Relative humidity (RH) in the jars was determined by measuring the RH of the room in which the jars were sealed and by calculating the increase in RH from the addition of known quantities of water on filter-paper strips.

Each determination for a given concentration of propylene oxide or for a certain RH level consisted of three jars or flasks, each containing three spore strips. At a pre-determined time each flask was opened and each of the three spore strips assayed. The results for the three strips from the same jar were averaged and plotted with the results from the other two jars on semilogarithmic graph paper. The rationale for analysis of these graphical plots was detailed by Phillips (1949). From these plots was determined the time for 90% kill (t_{90}), which is the reciprocal of the death rate constant, k, as explained by Phillips (1949). Each determination was run in duplicate, and the t_{90} values were averaged unless an obvious discrepancy between the two values was apparent. Additional determinations were performed to eliminate any such discrepancies.

Treatment of flaked or powdered foods. Most of the food materials were treated in the 4-L Case anaerobic jars, which were adapted to simulate a revolving blender. The jars were fitted with a sievelike expanded aluminum divider. The jars with their contents were placed horizontally on rollers and turned at 3 rpm within a bacteriological incubator. The propylene oxide was admitted as a gas through a valve opening from a warmed tube. A vacuum of 15–30 in. Hg was drawn before the propylene oxide was admitted. No water was added to any of the materials, but moisture determinations were performed on all foods to assay the amount of moisture available to react with the propylene oxide.

After an acceptable decontamination cycle had been developed with small quantities of food in the blender jars, pilot-scale cycles were performed in a 4-cu-ft glass-lined dryer-blender (Pfaudler-Permutit Co., Rochester, N. Y.), which had been modified to serve also as a revolving sterilizer with speeds of 3–15 rpm. The procedure developed with the experimental blender jars was followed although slight changes in time, temperature, or concentration of gas were occasionally necessary. Since this blender was water-jacketed, more flexibility in temperature control was available than with the experimental jars. Removal of the sterilizing gas was also easier since a constant vacuum could be pumped at the same time that the material was being tumbled. Sterile air rinses of the treated powders could also be accomplished. The maximum usable space in the 4-cu-ft blender was 2.5 cu ft, which would hold 50–60 lb of powder.

Since the level of natural contamination of certain microbes was too low to yield good quantitative data on the effect of treatment, e.g., the reduction of thermophiles from 500/g to less than 10/g, it was necessary to add organisms to a level of 10^4–10^5/g and follow by plate-count assay the reduction to 10^2 organisms/g. This inoculation procedure was used during the initial developmental phases of the treatment of a food. When an effective treatment cycle was developed (reduction of inoculated flora by 1 to 2 logarithms), this cycle was employed for reduction of the natural contaminants in that dried food.

Acetone-dried spores from washed, heat-shocked suspensions of *B. subtilis* var. *niger* (heat-shocked at 65°C for 30 min) or *Bacillus stearothermophilus* strain 1518 (heat-shocked at 100°C for 5 min) were used to increase the mesophilic or thermophilic counts of foods, respectively. Mold counts were increased by the addition of air-dried conidiospores of *Aspergillus niger* ATCC 6275. Spray-dried cells of *Serratia marcescens* (Bruch, 1958) were used to simulate coliform contamination.

Some storage tests were performed on the microbicidal effect of residual propylene oxide in treated foods. In these trials the material was added to the blender, and a vacuum of 25–30 in. Hg was drawn. After the vacuum was held for one min, the propylene oxide was drawn in as a gas from a warmed flask. The vacuum dropped to 15–20 in. Hg and was held during the decontamination cycle (1–3 hr). This phase was followed by a vacuum treatment for 5 min at 25–30 in. Hg. The blender was stopped and opened, and the material was dispensed and sealed in pint jars or polyethylene bags of 3-mil thickness.

At each sampling period two samples of each type of container and one control of each type of container for each temperature were tested. Fresh,

unopened samples were used at each sampling time. In addition to the microbiological tests, pH and residual epoxide were determined for some samples, but these data are not reported.

All spore strips and all samples of food were blended in small, sterile, Waring blender cups with sterile water prior to microbiological assay. Spores of *B. subtilis* var. *niger* from the paper spore strips were assayed on a tryptose agar medium (Bruch, 1958). Tryptone-glucose-yeast extract agar medium (Difco Laboratories, Detroit, Michigan) was utilized to determine the spores of this organism in food samples and for the bacterial (mesophilic) counts of all foods. Dextrose-tryptone agar medium (Difco Laboratories, Detroit, Michigan) was used to assay spores of *B. stearothermophilus* and for the thermophilic counts of all foods. Mold counts were made with mycophil agar medium (Baltimore Biological Laboratories, Baltimore, Md.) adjusted to pH 4. The plates for bacterial counts were incubated at 32°C, the plates for thermophilic counts at 55°C, and the plates for mold counts at room temperature.

The propylene oxide used was obtained from Distillation Products, Inc., Eastman Kodak Co. No attempt was made to inert the propylene oxide with carbon dioxide or halogenated hydrocarbons to prevent explosions. The equipment used was spark-proof. In addition, the drawing of a vacuum in many of the treatment cycles brought the propylene oxide concentration in the chambers above the upper explosive limit of 21.5% by vol. Table 1 lists the salient properties of propylene oxide.

RESULTS AND DISCUSSION

Since the literature contains no time-concentration data for the sporicidal properties of propylene oxide, various concentration were tested for different periods against a constant level of bacterial spores. Spore numbers were reduced by one logarithm (t_{90}) in 1 hr at 1250 mg/L (Table 2). If a first-order reaction is assumed, it would take about 6 hr to achieve sterility of the spore strips with 10^6 spores/strip at this concentration of propylene oxide.

The time required for 90% kill is lessened as the concentration of gas increases. If the concentration of gas is multiplied times the t_{90} value, a product is obtained that appears to be near a constant for the concentrations tested. Thus, the coefficient of dilution is near unity. If the concentration of gas is doubled, the time for 90% kill is approximately halved. The last value in Table 2 indicates that organic matter, such as food, in the sterilizing system can alter the time-concentration values for the destruction of a microorganism by gaseous propylene oxide.

After an effective time-concentration relationship had been established, the effect of RH on this relationship was determined. The data in Table 3 show that propylene oxide is more active at lower RH, as Kaye and Phillips (1949) found for ethylene oxide. This result also agrees with procedures for ethylene oxide sterilization in the food industry since no water is added to the dried materials to be decontaminated (Griffith and Hall, 1938, 1940a, b, 1942, Mayr and Kaemmerer, 1959; Rauscher *et al.*, 1957). It was assumed that any food product with 2–8% moisture had enough water

Table 1. Properties of propylene oxide.[a]

Molecular weight	58.08
Boiling point	33.9°C
Freezing point	−104.4°C
Flash point (Cleveland open cup)	−37°C
Specific heat	0.51 cal/g/°C
Heat of vaporization	160 Btu/lb
Specific gravity (apparent) at 20/20°C	0.8304
Solubility in water at 20°C	40.5% by wt
Explosive limits in air at 760 mm (upper)	21.5% by vol
(lower)	2.1% by vol
Maximum gas concentration at 34°C (760 mm)	2300 mg/L

[a] From Curme and Johnston (1952).

Table 2. Effect of gaseous propylene oxide (PPO) concentration on time for 90% kill of dry spores of *B. subtilis* var. *niger*.

PPO concentration [a]		Time for 90% kill (t_{90})	Ct_{90}
mg/L	mM/L	(hours)	(mM/L × t_{90})
410	7.1	2.4–3.6	17.0–25.4
830	14.3	1.6–2.0	22.8–28.5
1160	20.0	1.0–1.1	20.0–22.0
1250 [b]	21.5	1.0–1.1	21.5–23.7
1075 [c]	18.5	1.4–1.6	26.9–29.6

[a] Temperature was 36–38°C; relative humidity was 80–90%.
[b] Experimental chambers in this experiment were 4-L Case anaerobic jars. Rest of determinations were made with 2-L vacuum flasks.
[c] Twenty-five grams of cereal flakes were added to flasks.

Table 3. Effect of relative humidity on the sporicidal activity of gaseous propylene oxide against *B. subtilis* var. *niger*.

Relative humidity [a] (%)	Time for 50% kill (t_{50}) (hours)	Ct_{50} (mM/L × t_{50})
22	0.65	14.0
36	0.75	16.1
86	1.05	22.6
saturated	1.2–1.3	25.8–28.0

[a] Concentration of propylene oxide was 1250 mg/L (21.5 mM/L); the temperature was 36–38°C. The exposure chambers were 4-L Case anaerobic jars.

available for efficient treatment with propylene oxide.

The next determination should have been the effect of temperature on the rate of kill. Kaye and Phillips (1949) found that the temperature coefficient for the rate of sterilization with gaseous ethylene oxide varied between 2 and 3 per 10°C rise for both low- and high-RH runs. It was accepted that the time-concentration products (Ct_{90} values) for propylene oxide decontamination of the spore strips would be less at higher temperatures. Since the goal was the treatment of foods with propylene oxide, it was assumed that the effects of temperature on the microbicidal properties of propylene oxide could be gauged in foods decontaminated with this gas at various temperatures.

The first food to be treated with propylene oxide was flaked baby cereal that had been inoculated with a high concentration of dried spores of *B. subtilis* var. *niger*. The results (Table 4) show that destruction of the spores in a food does not go to completion but trails off to a constant level of survivors. Time-concentration products (Ct_{90} values) were calculated, but these values were very large and became meaningless with the trailing-off of the rate of spore destruction before 90% kill was achieved. This effect of organic materials on the Ct_{90} values was noted in Table 2 with the addition of cereal flakes to the jars containing spore strips. The presence of the food not only offers physical protection to the organism, but the food must physically absorb and chemically react with the oxide, thereby effectively reducing the gas concentration. The remaining data in Table 4 show that propylene oxide is much less active in food materials than is an equivalent concentration of ethylene oxide or equivalent concentrations of ethylene and propylene oxide (based on equal number of epoxy groups for each total concentration).

Reduction of the natural bacterial and thermophilic flora of a sample of cocoa powder was attempted. The results (Table 5) show the trailing-off phenomenon of reduction of count with time of exposure. At 37°C about 30–50% of the bacteria survived 3–6 hr at the concentrations tested. Destruction of the bacteria and thermophiles was greater at 55°C and with increased holding times at both temperatures.

Table 6 presents the results of treating several different materials in the 4-cu-ft re-

Table 4. Sporicidal activity of gaseous propylene oxide (PPO) and/or ethylene oxide (ETO) on dry bacterial spores mixed in flaked baby cereal.[a]

Epoxide concentration		Exposure system [b]	Temperature (°C)	Time (hr)	% survival of spores [c]
mg/L	% of wt of flakes				
PPO-1000	8	Static	37	3	3.7
PPO-1000	8	Static	37	4	5.2
PPO-1000	8	Static	37	17	0.14
PPO-1000	8	Static	37	60	0.16
PPO-1000	2	Dynamic	55	4	3.2
ETO-760	1.5	Dynamic	55	4	0.00009
PPO-500 + ETO-380	1.75	Dynamic	55	4	0.076
PPO-750 + ETO-190	1.88	Dynamic	55	4	0.76

[a] Per cent moisture of flakes = 3–4%.
[b] Two-liter vacuum flasks were used in the static experiments. The dynamic system utilized 4-L Case anaerobic jars.
[c] The spores of *B. subtilis* var. *niger* were at a level of 2.5 × 10⁷/g of cereal flakes.

Table 5. Reduction of the microflora of cocoa with gaseous propylene oxide (PPO) in 4-L blender jars.

PPO concentration		Temperature (°C)	Time (hr)	% survival[a]	
mg/L	% wt of cocoa			Mesophiles	Thermophiles
1000	4	37	1	75	.....
			6	45	34
2500	1.7	37	3.5	30	6.3
			24	1.6	3.2
2000	2	37	3	51	.....
2000[b]	2	37	4	10	50
1000	2	55	5	2.8	4.4
			6	1.9	2.2
3000	2	55	6	10	11
			19	4.2	3.1

[a] Average control counts per gram: mesophiles, 9×10^4; thermophiles, 3×10^4.
[b] Sample of cocoa from different manufacturer: this sample had 8% moisture. Other five samples were from same manufacturer and had moisture levels of 2–4%.

volving blender. Before any foods were treated, tests were run with spore strips and small samples of inoculated cereal flakes in plastic and paper bags. The greater resistance to propylene oxide by spores of *B. subtilis* var. *niger* than by spores of *B. stearothermophilus* had been noted in some preliminary screening tests with these organisms in the blender jars. Survival of the mold spores in the bagged samples was due to the inability of the propylene oxide to pass rapidly through the plastic bags and penetrate the cereal flakes; later tests showed complete destruction of mold spores in actively tumbled bulk samples at similar time-temperature-concentration relationships.

The data from the treatment of powders and flakes in the 4-cu-ft blender show a rapid destruction of vegetative cells and mold spores. The bacterial and thermophilic survival levels in the cocoa are in agreement with the results in Table 5, confirming reduction of these organisms by about one logarithm at practical time-concentration values (3 hr at concentrations based on 2% by wt).

When it became apparent that 24 hr or longer would be necessary for reduction of 2 or 3 logarithms in the bacterial count of cocoa, experiments were set up to determine the effectiveness of various ways of adding propylene oxide and storing the cocoa. In the first experiment (Table 7) the propylene oxide was pipetted directly into each bag or bottle of cocoa. The bacterial count in the cocoa in the bottles was reduced 3 logarithms after 2 days' exposure. No further decrease occurred during the next 19

Table 6. Reduction of microbial flora of powders with gaseous propylene oxide (PPO) in a 4-cu-ft revolving blender.

Material	PPO concentration		Time (hr) and temp (°C)	Contamination per gram	% survival
	mg/L	% wt of powder			
Spore strips[a]	1450	.....	2.5/30	*B. subtilis* var. *niger*–10^5	0.7
				B. stearothermophilus— 5×10^5	0
Cereal flakes in paper and plastic bags				*A. niger*—2×10^3	20–40
Yeast powder (6% moisture)	1720	2	3/38	*S. marcescens*—1×10^3	0
Protein flakes (14% moisture)	500	2.8	0.5/20	*S. marcescens*—4×10^2	10
Cocoa (4.5% moisture)	700	2	1/38	Mesophiles—10^5	55
				Molds—10^4	0.7
				Thermophiles—10^3	42
Cocoa (8% moisture)	1200	2	2/38	Mesophiles—10^5	14
				Molds—2×10^2	12
				Thermophiles—2.7×10^3	19

[a] Blender was used as a static chamber in this trial.

days. The kills at 55°C were on the same order of magnitude as those at 37°C. Because of the higher bacterial count of the samples from the polyethylene bags, it appears that some of the gas escaped through the plastic.

The odor of propylene oxide was detected in the Mason jars throughout the 21-day storage period, but was gone after 2–5 days in the plastic bags. All of the samples treated with a concentration of ethylene oxide that had one-eighth of the number of epoxy groups as the propylene oxide concentration had lower counts than the propylene-oxide-treated samples. This result indicates that propylene oxide lacks the chemical reactivity and the penetrating ability of ethylene oxide.

In the second experiment (Table 8) the same amount of propylene oxide on a weight basis, but lower on a volume basis than in the first experiment, was mixed with the cocoa in the 4-cu-ft blender. After 1 hr a brief vacuum cycle of 28 in. Hg was drawn, and the cocoa was then bottled or bagged. The results show only a modest decrease in bacterial count after 3 days' storage over the initial reduction of the microbial population after treatment in the blender. Reduction of mold count was greater than 99% after the one-hour blender treatment, and no further decreases were noted. It appears that the residual propylene oxide in the cocoa after treatment in the blender was insufficient for significant microbial destruction. As in the first storage experiment, a lower concentration of ethylene oxide gave the same order of reduction of the microbial flora as did a much higher concentration of propylene oxide.

The third experiment (Table 9) was similar to the second except that blender jars were used and the decrease in thermophiles was followed, as well as that of molds and bacteria. Approximately 99% of the added thermophilic spores were destroyed in the 3-hour treatment in blender jars with propylene oxide. Storage for 2 days gave a further drop in count of one logarithm. The results for destruction of bacteria and molds are similar to those given in Table 8. All of the samples treated with ethylene oxide showed greater reduction of microorganisms than the propylene oxide-treated samples.

From these data it becomes apparent that propylene oxide can function as a gaseous decontaminant for certain groups of microorganisms in flaked or powdered foods. It cannot function as a gaseous sterilant for dried foods, as can ethylene oxide. Propylene oxide will adequately destroy molds and coliforms to meet most food specifications. Reduction of thermophiles to acceptable levels can be achieved with reasonable process cycles with many foods. If reduction of the bacterial (mesophilic) count of greater than 90% is desired, propylene oxide will have to be in contact with the product for long periods.

Most of the data presented in this paper were obtained under dynamic cycles of treatment since it was noticed in early screening experiments that small static samples inoculated with bacterial spores resisted decontamination by propylene oxide during contact times of 1–6 hr. Adding liquid propylene oxide to a static sample such as a small package of cereal or cocoa may give adequate decontamination after storage for several days, but destruction of the food that comes into actual contact with the liquid epoxide will be noted. It is doubtful, though, that the addition of liquid propylene oxide to one end of a 100-lb bag of powdered food will give adequate microbial decontamination of the contents. Decontamination of this same bag of powdered food by gaseous propylene oxide under static conditions in a chamber does not appear possible from the results obtained in this study.

Treatment of food with an epoxide will result in chemical modifications of some constituents, as has been shown by radioactive tracer techniques (Gordon *et al.*, 1959), as well as residuals of the epoxide and its homologous glycol. With proper treatment and storage the amount of residual propylene oxide will drop rapidly. The residual propylene glycol can be determined and offers no toxicity hazard. The possibility exists that the alkylation of food constituents may result in toxic intermediates, but there has been no evidence of such toxicity to date from foods treated with ethylene oxide or propylene oxide. The chief problem with foods treated with epox-

Table 7. Reduction of bacterial flora of cocoa during storage with propylene oxide (PPO) or ethylene oxide (ETO) in bottles and plastic bags.

Days of contact	% survival of bacteria [a]			
	37°C		55°C	
	PPO—2% [b]	ETO—0.2%	PPO—2%	ETO—0.2%
In bottles				
2	.15	.22	.32	.15
5	.10	.02	.23	.08
12	.29	....	.70	....
21	.29	....	.28	....
In polyethylene bags				
2	8.1	1.7	.79	.25
5	4.6	1.3	1.4	.12
12	1.0	1.8	.62	....
21	2.1	....	.72	....

[a] Average control counts for 6 samples taken during 21 days storage at each temperature: 37°C = 1.3×10^5 bacteria/g; 55°C = 9.1×10^4 bacteria/g.
[b] The propylene oxide was added at the rate of 3 g/150 g of cocoa in a pint jar or plastic bag; the ethylene oxide was added at the rate of 0.3 g/150 g.

Table 8. Reduction of microbial flora of cocoa with propylene oxide (PPO) and ethylene oxide (ETO) in the 4-cu-ft blender followed by storage in bottles and plastic bags.

Days of contact	% survival at 37°C [a]			
	Bacteria		Mold	
	PPO—2% [b]	ETO—0.2%	PPO—2%	ETO—0.2%
In bottles				
.04	55	59	.7	3.3
3	22	15	....	....
7	15	12	.2	.05
In polyethylene bags				
.04	55	59	.7	3.3
3	27	32	....	....
7	35	28	.2	.1

[a] Average control counts for 2 samples during 7 day storage: bacteria (mesophiles) = 1.3×10^5/g; mold = 3.0×10^4/g (spores of *A. niger* added).
[b] The propylene oxide was added at the rate of 2% by wt or 700 mg/L by vol; the ethylene oxide was added at the rate of 0.2% by wt or 200 mg/L by vol.

Table 9. Reduction of microbial flora of cocoa with propylene oxide (PPO) and ethylene oxide (ETO) in blender jars followed by storage in bottles.

Days of contact	% survival at 37°C					
	Mesophiles		Mold [a]		Thermophiles [a]	
	PPO—2%	ETO—0.5% [b]	PPO—2%	ETO—0.5%	PPO—2%	ETO—0.5%
.12	62	3.8	....	.05	.94	.005
1	....	....	.03	.03	.079	.001
2	23	3.8	.01	0	.058	.001

[a] Average control counts for 2 samples: mesophiles = 1.3×10^5/g; mold = 6.8×10^4/g (spores of *A. niger* added); thermophiles = 1.9×10^6/g (spores of *B. stearothermophilus* added).
[b] The propylene oxide was added at the rate of 2% by wt or 2000 mg/L; the ethylene oxide was added at the rate of 0.5% by wt or 300 mg/L by vol.

ides is the decrease in the nutritional value of the food.

Alteration of the nutritional value of an epoxide-treated food is centered in the destruction of vitamins such as thiamine, niacin, pyridoxine, riboflavin, and folic acid (Bakerman *et al.*, 1956, Windmueller *et al.*, 1959a), and of amino acids such as histidine, methionine, cysteine, and lysine (Windmueller *et al.*, 1959b). Every food product that is to be treated with propylene oxide will have to be checked either by chemical or microbiological or feeding tests to assure that the nutritional value has not been unduly changed. (The Food and Drug Administration should be consulted on matters relating to the treatment of food with propylene oxide.)

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Studies on the Ecology of Selected Food Poisoning Organisms in Foods. I. Growth of Staphylococcus Aureus in Cream and a Cream Product

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SUMMARY

A commercially produced whipped cream product, sold as a "cream puff" from unrefrigerated home delivery trucks, was investigated. The need to provide refrigeration of cream and cream-filled products on trucks during the hot weather in southern California was one of the basic considerations. The product investigated was one of the most hazardous food-poison-producing food products. Although over a million cream puffs had been sold in southern California from these trucks, no report of food poisoning was recorded, or even a claim against the company of suspected food poisoning. The laboratory investigation did not explain why there have been no food poisoning cases reported from eating this product.

Although public health workers generally assume that staphylococcus food poisoning is an important public health problem, only 62 outbreaks (involving 2,291 persons) were reported by the U. S. Public Health Service in 1958 (Dauer and Davids, 1958). Most if not all of these could have been prevented by proper temperature control of the food after cooking. Lack of accurate reporting is generally given as the reason we don't have more food poisoning cases reported than are now officially recognized. Undoubtedly, a great many cases go unreported for one reason or another; however, in spite of the presence of all the proper conditions for production of toxin and consequent food poisoning, poisoning may not arise because of other factors, some of them possibly inherent in the food. One factor, the effect of naturally occurring microorganisms in foods on the growth of enterotoxigenic staphylococci, has not been extensively investigated.

Mossel and Ingram (1955) presented an excellent review of factors influencing selection of the dominant organisms in food spoilage, one of which is antagonism, either through changes in the substrate or by the production of antibiotic substances. Studies on the multiplication of *Staphylococcus aureus* in raw (Jones *et al.*, 1957; Smith, 1957) and pasteurized (American Public Health Asso., 1958) milk have indicated that the normal inhabitants of milk tend to suppress growth of this organism in accordance with the number of both *S. aureus* and other organisms initially present. Smith (1957) suggested that (other conditions being favorable) a very clean product would be more likely to cause food poisoning than one in which the standards were much lower. Other workers have demonstrated direct antagonism between *S. aureus* and *Escherichia coli* (de Lavergne *et al.*, 1955; Parmala, 1956) and between other organisms (Charlton, 1955) that may be found in milk.

The objective of this series is to study the effect of the normal bacterial inhabitants of foods on the growth of food poisoning organisms. The investigation reported in this paper was designed to observe the growth of a coagulase-positive *S. aureus* strain in the presence of the normal flora of a pasteurized cream product. Since conditions of manufacture and delivery to the consumer also play a very real role in the occurrence of food poisoning, an attempt was made to simulate these conditions in the laboratory.

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Various techniques were examined to obtain the desired data.

MATERIALS AND METHODS

Product and sampling. The cream product selected for study was manufactured locally. It was composed of 36% grade A pasteurized cream, a paste mixture of starch, sugar, and gelatin, and 0.064% sodium benzoate whipped together to form a fill, which was then injected into a baked pastry shell. All samples were collected aseptically in appropriate sterile containers at the hour of manufacture and transported to the laboratory for study: 1) Paste was collected after the ingredients were mixed; 2) shells were collected immediately upon leaving the oven; 3) cream was obtained prior to the mixing of the fill; and 4) fill samples were collected immediately after the ingredients were mixed.

Bacteriology of the components. To obtain some information on the natural bacterial flora of the product and its various ingredients, a series of tests were performed. Included were total counts, coliform Most Probable Numbers (MPN), enterococcus MPN's, Salmonella-Shigella presence, and counts of organisms in the shell and paste by an MPN method, using brain-heart infusion broth. The methods of performing these tests are described elsewhere (American Public Health Assoc., 1958). Representative colonies from the total count plates were transferred to appropriate agar slants for further identification. Isolates were examined in accordance with the Manual of Microbiological Methods (1957) and identified by reference to Bergey's Manual (Breed *et al.*, 1957).

All samples came from the same dairy and were presumably handled in the same manner, resulting in similar kinds of organisms in the cream on different days. The numbers of organisms were fairly constant in both fill and cream, usually 1,000–10,000 organisms per ml. The paste was also handled in a uniform manner.

Preparation of samples. The product was a froth or whip composed of starch, cream, and air, which made volumetric sampling procedures difficult. To eliminate this, the product was allowed to melt at 36°C for ½ hr. It is assumed that this change in structure did not greatly affect the growth characteristics of *S. aureus*, since at temperatures of good *S. aureus* growth (30–36°C) the product rapidly melted to a fluid state. The subsequent liquid was measured volumetrically into three sterile 125-ml Erlenmeyer flasks. The cream was measured volumetrically into three other sterile flasks. One flask of each was inoculated with the test organism, another of each served as uninoculated controls, and the remaining two were autoclaved 15 min at 121°C and then inoculated with the test organism.

Culture. The *S. aureus* culture used in the growth studies was a coagulase-positive gelatinase-negative strain of phage type 42D/81 obtained from the California State Department of Health. Preliminary studies showed this organism to be distinguishable from the normal salt-tolerant organisms growing on the Chapman-Stone medium. A suspension of organisms equivalent to a McFarland turbidity standard number 1 was prepared by washing a 24-hour-old agar slant with 0.1% peptone water (used in making all dilutions). A volume of an appropriate dilution was added to the sample flasks to yield 15–150 organisms per ml of sample.

Plating technique. Size of inoculum, counts of fresh cream and fill prior to inoculation, and counts of cream and fill (both inoculated and uninoculated) after 24 hours of incubation were determined by a drop-plate technique adapted from Mallmann and Broitmann (1956) on trypticase soy and Chapman-Stone agars. Preliminary studies with a pure culture of *S. aureus* indicated that this technique gave the same results on both these media. In practice, up to eight dilutions could be made, each in triplicate, on a single plate using 0.01-ml samples. Tellurite glycine agar considerably inhibited the test organism, and therefore was not used further. Sampling of colonies on Chapman-Stone agar indicated that they were staphylococci.

Simulated summer delivery temperatures. An experiment designed to simulate summer delivery conditions was performed as follows: Fill was prepared as previously described, and inoculated with *S. aureus*. In accordance with the actual procedure of product manufacture and delivery, samples were immediately left 20 hours in the refrigerator and then incubated 2 hours at 25°C, 2 hours at 30°C, and finally 6 hours at 35°C. Counts were made, as previously described, at the end of 2 and 6 hours of incubation at 35°C.

RESULTS AND DISCUSSION

Although cream products of various kinds have been reported as vehicles of staphylococcal food poisoning (Dack, 1956), the product under discussion, considered potentially dangerous by public health authorities, has not been officially reported as the cause of any cases of food poisoning, in spite of being delivered and sold without refrigertion in the heat of the summer.

Some foods seem characteristically involved more often in outbreaks than others.

One could advance several possible explanations, including suitability of the food as a substrate for staphylococci, and temperature of preparation and holding (Dack, 1956). To these classical reasons might also be added high initial contamination or growth of other organisms before introduction of the staphylococci (Jones *et al.,* 1957; Smith, 1957; Takahashi and Johns, 1959) and outright competition or antagonism by organisms growing in the product (Mossell and Ingram, 1955) One might expect these last two factors to be more important in foods that are cooked only partially before being served. The growth of *S. aureus* in cream and fill bears some relationship to the above factors.

Cream and fill experiments were performed in pairs since the particular batch of cream was used in making the corresponding fill. All experiments corresponded to one or the other of the pairs of results reported in Fig. 1. Initial bacterial counts were low (1,000 per ml) in half of the experiments, and high (100,000 per ml) in the other half. The normally occurring staphylococci were similarly low and high in each instance. The samples with the lower initial staphylococcus counts seemed to have the greater increase after 24 hr of incubation (Fig. 1-A, C). However, based on the great increase in numbers of other cream organisms, the normally occurring staphylococci did not appear to multiply as much as would be expected. In fill (Fig. 1-B, D), the staphylococci grew much more abundantly during the incubation period. This difference between fill and cream may be due to several factors: addition of more readily available nutrients; dilution of some natural inhibiting factor of cream (Claydon *et al.*, 1958); reduced competition or antagonism of other organisms; and the possibility that 0.064% sodium benzoate disturbed the ecological balance sufficiently to permit greater growth of the staphylococci even though this chemical is not recognized as very effective at neutrality (Frazier, 1958). It has not yet been determined which of these are operative.

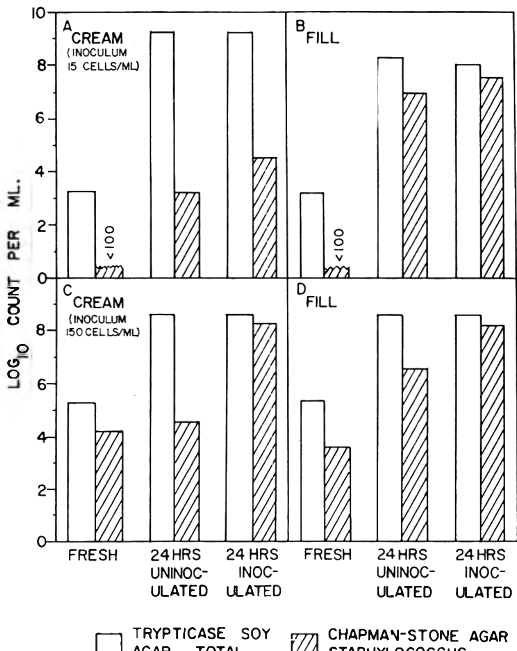

Fig. 1. Counts of *Staphylococcus aureus* and total counts from two representative pairs of growth experiments in cream and fill.

The *S. aureus* inoculated into cream or fill showed a variable growth pattern—except in the autoclaved cream and fill samples, in which the test organism yielded 24-hour counts of about 1.5×10^8 in all cases. For this reason these controls are not included in Fig. 1 or 2. In an attempt to represent the natural conditions of contamination, the inoculum was 15 organisms per ml of product for the experiments reported in Fig. 1-A,B, and 150 in Fig. 1-C,D. There is little doubt that these are higher than normally encountered and it is recognized that lower levels may increase the apparent competition. The test organism grew equally well in all fill experiments and seemed to indicate that the same factors that influenced the growth of the normal staphylococci were operating. In cream, however, the results were quite different. Where the inoculum was high (Fig. 1-C), even though the initial total count was also fairly high, the test organism grew well—as well as in the fill and autoclaved controls (1.5×10^8 per ml). The large size of the inoculum may have been the controlling factor, but it may also have been only partially controlling in that the naturally occurring staphylococci may have laid the groundwork for overcoming any competition

Table 1. Range of the numbers of various groups of organisms found in the ingredients of the cream product.

Determination	Source	Most-common range
Total count	Cream	1,000–10,000/ml[a]
Total count	Fill	1,000–14,000/ml
Total count (MPN)	Paste	78–100/100 ml
Total count (MPN)	Shell	100–200/shell
Coliform MPN	Cream	7.8–23/100 ml
Enterococcus MPN	Cream	20–78/100 ml[b]
Salmonella-Shigella	Cream	none

[a] Extreme values of 122,000 and 2,400,000.
[b] Extreme values of 0 and 1300.

present. Where the inoculum was smaller (Fig. 1-A), the test organism grew poorly in competition with the other organisms present. Similar results have been reported (Smith, 1957).

Table 1 lists the groups of organisms found in the product and its ingredients. The coliform, enterococcus, and *Salmonella-Shigella* tests on paste and shell were negative. The fill results corresponded to those obtained from cream. The coliform organisms isolated from the cream were fairly evenly distributed between typical *Escherichia coli*, *Aerobacter aerogenes*, and Intermediates as determined on eosin methylene blue agar. Isolates from the enterococcus tests proved to be *Streptococcus faecalis*.

A total of 64 representative colonies were isolated from the product and identified. These organisms are listed in Table 2. This list is not intended to indicate frequency of the particular organism in the product. The studies indicated that virtually all the organisms in the product came from the cream (except a few heat-resistant spore formers).

A large number of the Gram-positive organisms, reported to be heat resistant, could have survived pasteurization, although no heat resistance tests were performed. The remaining Gram-positive and all of the Gram-negative bacteria probably arose from post-pasteurization contamination — from equipment, air, hands, etc.

Heineman (1957) states that few if any salt-tolerant yellow micrococci survive pasteurization. If this is generally true, the recovery of these organisms from the product assumes some importance. In Table 2 it can be seen that five yellow staphylococcal strains were isolated from the product. Two of these were coagulase-positive, and therefore are suspected food-poisoning organisms. This does not mean that these organisms are capable of producing enterotoxin but it does mean that organisms of the type that can cause food poisoning do find entrance into the product during production and are capable of growth under the proper conditions. The organisms surviving the shell baking (218°C maximum for ½ hour) and paste manufacture (boiling for 5 min) were tentatively identified as spore-forming *Bacillus subtilis*. These few organisms did not materially add to the large numbers introduced from the cream.

One finds numerous reports on antagonism and even production of antibiotics (certain *Pseudomonas*, coliform, and *Bacillus* species) by strains of bacteria closely resembling those recovered from the product. It appears that some competition is encountered from the normal inhabitants of

Table 2. Organisms recovered from the cream product.

Genus—species	No. of strains isolated
Gram-positive	
Staphylococcus aureus yellow[a]	5
S. aureus white	4
Sarcina sp.	5
Streptococcus faecalis	2
S. lactis var. *cremoris*	3
Bacillus (subtilis)[b]	4
B. (cereus)	2
B. (megatherium)	2
B. coagulans	1
B. sp.	2
Heterogeneous rods—no spores	4
Gram-negative	
Aerobacter aerogenes	13
A. cloacae	3
Escherichia freundi	1
(Aeromonas) sp.	2
Pseudomonas (fluorescens)	2
Ps. (xanthe)	1
Ps. sp.	7
Yeast—unidentified	1
Total	64

[a] Two were coagulase-positive strains.
[b] Parentheses indicate a tentative identification.

the product, but whether antagonism or antibiotic production plays a role in *S. aureus* growth inhibition, and what organisms might be involved, has not yet been determined. Investigation of these possibilities is under way.

A consideration in any discussion of the growth of staphylococci in foods is the problem of how many organisms it takes to elicit sufficient enterotoxin to produce the typical syndrome of food poisoning. Although the number of organisms is unknown and probably varies from food to food, Jones *et al.* (1957) reported that experience suggests 10 million cells per ml in milk. The epidemiological observations of one of the present authors (AHB) indicate a similar experience. Reference to Fig. 1 shows that the fill inoculated with *S. aureus* meets this figure and that the normal staphylococci in fill should, from a practical standpoint, be included also. The product would appear to be able to support growth of sufficient organisms and produce enough toxin to cause food poisoning under *this condition* of incubation (36°C for 24 hours). To elucidate this point, simulated delivery-truck experiments were performed.

The product itself, based on the bacterial count of the cream, would be considered an excellent product. It was handled as little as possible, it contacted only thoroughly sanitized equipment, all ingredients had been refrigerated since compounding, and the finished products were refrigerated for about 12 hours immediately after the shell was filled (mechanical). They were then taken out of the refrigerator, and placed at room temperature until picked up by the delivery trucks. On the truck the product was placed on the unrefrigerated bottom shelves and remained there until sold. The excessively hot summers in southern California prompted the laboratory use of what was considered extreme conditions of heating. The maximum temperature used in the study was 35°C (95°F). The period of warm-up to, and the incubation period at, this temperature were considered somewhat severe even for this locality.

Fig. 2 gives the results of two experiments performed in accordance with the simulated times and temperatures. The initial inoculum in both cases was the same—15 organisms per ml of product. Initial total counts were not performed.

Although one cannot base too much reliance on the 10-million-cell food-poisoning factor, it can be seen that in neither experiment did growth reach this figure, though the 6-hour counts in run 2 were very close. In all fairness to the product, the 6-hour figure represents the time at which the truck returned to the plant. Most of the product was sold early in the day, at least by the time 2 hours at 35°C had passed. The left-hand column of each pair in Fig. 2 more closely corresponds to the time of actual sale, and only in run 2 did growth approach significance at this time. The close similarity of inoculated and uninoculated samples can not be explained at this time.

All products held under condition of simulated delivery were rejected in consumer acceptance tests. The product began melting after 2 hours at 30°C, and at the end of the incubating period at 35°C the shell had soaked up the liquid, becoming very soggy and unappetizing in appearance. Products held 6–8 hours at 30°C had a mixed reception, some accepting and some rejecting. Only when held at temperatures below 30°C was the product completely acceptable. Consumer rejection probably plays a large role in the absence of reported cases of food poisoning from this product.

It was observed that the growth of *S. aureus* in this whipped cream product enclosed in a pastry shell is inhibited to some extent in the presence of naturally occurring

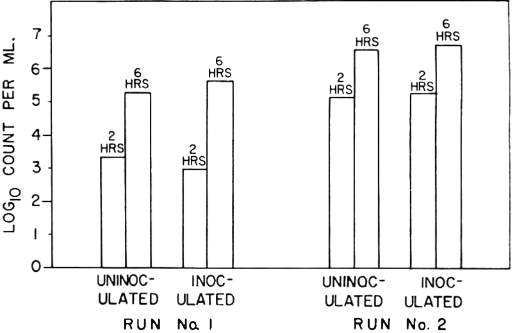

Fig. 2. Counts of *Staphylococcus aureus* after 2 and 6 hours of incubation at 35°C in the simulated delivery-temperature experiments.

competitors. It has not yet been determined how this is accomplished. Under laboratory conditions the test organism grows quite well and would probably cause food poisoning if toxin were formed. Under conditions of delivery of the product, however, times and temperatures of incubation are not as rigorous as laboratory conditions. Simulated delivery studies indicated that, at temperatures of good *S. aureus* growth, the product melted, and in this state would probably be unacceptable to the consumer.

It appears that, though the product can support the growth of *S. aureus* and is thus potentially dangerous, the development of food poisoning among consumers is dependent upon two factors in addition to the classical conditions (Dack, 1956) for initial enterotoxigenic *S. aureus* growth and temperatures suitable to growth: a) reduced competition from other organisms, and b) consumer acceptance of the product after it has been held under conditions permitting growth of *S. aureus*.

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The Carotenoids of Navel Oranges

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SUMMARY

The carotenoid mixtures of the peel and pulp of navel oranges were investigated. The composition of both is qualitatively much like those from Valencia oranges. About half of the total carotenoids in both pulp and peel consisted of violaxanthin and its 5,8-epoxide isomerization products. The peel carotenoids contained a fraction, possibly monol epoxides, in considerably greater quantity than found in Valencia orange or tangerine peels. This fraction included mono- and diepoxides of cryptoxanthin, and was the best source so far located for a cryptoflavin-like carotenoid previously found in several other fruits. Neoxanthin and a trolliflor-like pigment were found in the pulp of navel oranges.

In earlier work we investigated the carotenoids in Valencia orange juice (Curl, 1953; Curl and Bailey, 1954, 1955) and in Valencia orange pulp (the edible portion) and peel (Curl and Bailey, 1956) from California fruit. The composition of the carotenoid fractions of navel orange pulp and peel have now been investigated, with the object of ascertaining whether there are significant differences between the carotenoids of Valencia and navel oranges, and in particular whether navel oranges might be a better source of some of the carotenoids of incompletely known structure that are found in Valencia oranges. Although the carotenoid mixture from navel oranges was found to be qualitatively much like that of Valencias, the peel was found to be a considerably richer source of a cryptoflavin-like pigment previously found in much smaller amounts in several other fruits. Trans-neoxanthin was obtained from the pulp of navel oranges; it had not previously been isolated from any citrus fruit.

EXPERIMENTAL

The oranges used were obtained in March and early April from a local market. They were fully ripe and firm. Amounts used were 2,250 g of pulp and two lots of 750 and 1150 g of peels.

Extraction of pulp. Each batch of 250 g of pulp was blended with 250 ml of water and 6.6 g of magnesium carbonate, and the blend was mixed with an equal volume of methanol. After standing about 30 min, 13% by weight (of the fruit used) of filter aid (Celite 545[b]) was added, and the mixture filtered on a Buchner funnel precoated with filter aid. The filter cake was blended with acetone, and the extract processed as previously described (Curl and Bailey, 1959), including saponification. The saponified material was finally dissolved in benzene.

Extraction of peel. In batches of 150 g the peel was blended with 350 ml of water and 2 g of magnesium carbonate. To the combined blends was added 13.5% of filter aid, and filtered on a Buchner funnel precoated with filter aid. The filter cake was worked up essentially as previously described (Curl and Bailey, 1959), with one improvement. The residue from the evaporation of the acetone extract was transferred to a separatory funnel with water and ether, *ca.* 2 g of sodium chloride and methanol added (*ca.* 10% by volume of the aqueous solution). The addition of the methanol results in much more rapid and cleaner separations in the ether extraction, and is in general advantageous.

Countercurrent distribution. The pulp and the first peel extract were each divided into 2 equal parts. One-half of each extract was used in 100-transfer countercurrent distribution runs in a Craig apparatus with solvent system I (petroleum ether (Skellysolve B) and 99% methanol, 1.8:1 v/v) to separate the carotenoids into: (a) hydrocarbons, (b) monols, and (c) diols and polyols (Curl, 1953). The other halves were used in 200-transfer runs with solvent system II (benzene, petroleum ether, and 87% methanol, 1:1:1.15 v/v) to separate the carotenoids into: (a) hydrocarbons and monols, (b) diols, (c) monoepoxide diols,

[a] A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

[b] Mention of commercial products by name does not imply that they are endorsed by the Department of Agriculture or recommended over similar products of other manufacturers.

(d) diepoxide diols, and (e) polyols. The N_{100} value of several polyol constituents was determined in solvent system III (petroleum ether, acetone, methanol, and water, 1.25:1:0.1:0.65 v/v) (Curl, 1960).

The second peel extract was used in a 204-transfer run with system I, in order to separate the monol fraction more completely into two parts. The hydrocarbon and combined diol-polyol fractions were discarded.

Chromatography. The hydrocarbon and monol fractions obtained by distribution with solvent system I, and the diol, monoepoxide diol, diepoxide diol, and polyol fractions obtained with system II, were each chromatographed on columns of magnesia (Westvaco No. 2642) plus filter aid (1:1 v/v), 14 by *ca.* 160 mm, and the various constituents eluted by a graded series of eluants (Curl and Bailey, 1959). The two monol fractions obtained in the second peel extraction were chromatographed on columns of magnesia (Sea Sorb 43), 14 by *ca.* 80 mm, without a diluent, and using the same eluants as with magnesia 2642. In a few instances fractions obtained from the latter were rechromatographed on a column of calcium hydroxide (Moster *et al.*, 1952), 14 by *ca.* 70 mm, with the starting eluant being 5% ether (USP) in petroleum ether (dried to clarity over anhydrous sodium sulfate).

Spectral data for the various bands obtained on chromatography were obtained with Carey recording spectrophotometers models 11 and 14.

Hydrochloric acid test. In earlier work (Curl and Bailey, 1954), details were given for the hydrochloric acid–ether test for carotenoid epoxides. It was found that monol epoxides gave much paler tests than the corresponding diol epoxides, hence a modified test (Curl and Bailey, 1961) was used in the present work. In the test the sample solution was evaporated *in vacuo* and the residue dissolved in 4.5 ml each of ether and methanol; 1 ml of concentrated hydrochloric acid was added, and 1 ml of water to a duplicate control solution. Carotenoid diepoxides with this test usually change to a greenish blue, monoepoxides to a yellowish green. The color change sometimes occurs slowly so the solution should be allowed to stand for several hours.

RESULTS AND DISCUSSION

The total carotenoids, measured in an Evelyn photoelectric colorimeter at 440 mμ, and calculated as beta-carotene by means of a conversion table, were 23 and 67 mg per kg, respectively, for the pulp and peel. Previous results with California Valencia oranges were respectively 24 and 98 mg per kg (Curl and Bailey, 1956).

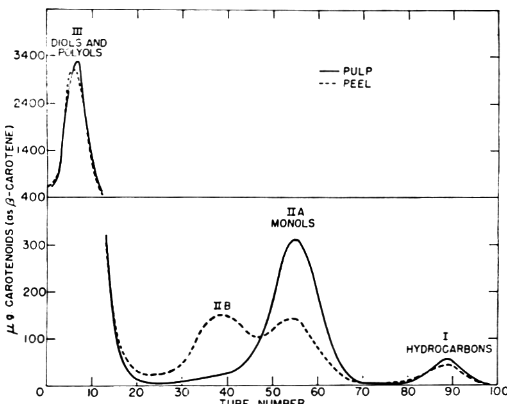

Fig. 1. Countercurrent distribution of the carotenoids from navel orange peel and pulp with the system petroleum ether and 99% methanol.

Countercurrent distributions. The distributions with system I are shown in Fig. 1. The curve for the pulp carotenoids showed a very large diol-polyol fraction, a much smaller monol fraction, and a very small amount of hydrocarbons. The N_{100} values (position of maximum per 100 transfers) of the latter two were 56 and 91, respectively, identical with those of cryptoxanthin and beta-carotene, respectively. The curve for the peel carotenoids was similar to that of the pulp carotenoids, except the monol fraction was about half as great, and it had an extra maximum of similar magnitude with N_{100} of 39. Fractions with a similar N_{100} value had previously been found in carotenoids from Valencia orange peel (Curl and Bailey, 1956), Ruby Red grapefruit peel (Curl and Bailey, 1957b), tangerine peel (Curl and Bailey, 1957a), and cling peaches (Curl, 1959) but in all of these cases they were considerably smaller than the monol fraction. The curve for the pulp carotenoids shows a minor inflection at about tube 39. This fraction probably consists of substances containing one hydroxyl group and a less hydrophilic group such as an epoxide.

The distributions with system II in both cases showed fractions with N_{100} value of 39, which amounted to over 50% of the total carotenoids. This value is in good agreement with that of violaxanthin, a diepoxide diol, which in system II was found to be 40.

Much smaller fractions with X_{100} values of 53–54 and 68–69 corresponded to monoepoxide diols and diols, respectively. In this system antheraxanthin had an X_{100} value of 55, and zeaxanthin and lutein of 69. Table 1 gives the fractional composition of the pulp and peel carotenoids based on the distributions in systems I and II.

Chromatography of fraction I. Table 2 gives the constituents found on chromatography of the several fractions, the first one eluted being listed first. The very low content of alpha- and beta-carotenes is noteworthy, the amounts of these substances being much less than phytoene, phytofluene, and zeta-carotene. An unusual constituent was noted in the pulp carotenoids with absorption maxima in petroleum ether at 418, 393, and 373 mμ. This substance (P418), which was present in very small amount, may be a cis-zeta-carotene.

Chromatography of fraction II. In the pulp carotenoids the principal constituent was cryptoxanthin. It was accompanied by much smaller amounts of a hydroxy-alpha-carotene-like substance, and of fractions that resembled cryptoxanthin 5,6-epoxide, cryptoxanthin 5,6,5′,6′-diepoxide, and cryptoflavin (cryptoxanthin 5′,8′-epoxide (Karrer and Jucker, 1946).

In the first run with the peel carotenoids, fractions IIA and IIB were combined. Because chromatography showed this fraction to be rather complex, a second lot of peels was worked up, and a 204-transfer run was carried out with system I in order to achieve a better separation of fractions IIA and IIB. The total amount of carotenoids used in the second run was about 3 times that of the first.

In fraction IIA from the peel, the principal constituent was cryptoxanthin. It was accompanied by much smaller amounts of two hydroxy-alpha-carotene-like bands. After treatment of these bands with hydrochloric acid in ether-methanol, the spectral

Table 1. Fractional composition of carotenoid mixtures from navel orange pulp and peel as determined by countercurrent distribution.

Fraction	% (as beta-carotene) Pulp	Peel
I (hydrocarbons)	2	2
IIA (monols)	18	8
IIB (monol epoxides ?)		9
IIIA (diols)	5	5
IIIB (monoepoxide diols)	17	9
IIIC (diepoxide diols)	53	60
IV (polyols)	5	6

Table 2. Carotenoid composition of navel orange pulp and peel.

Fraction	Constituent	Approx % of carotenoid mixture Pulp	Peel
I[a]	phytoene	4.3	12.1
	phytofluene	4.4	7.3
	alpha-carotene	0.12	0.03
	P418[b]	0.11	—
	beta-carotene	0.5	0.15
	zeta-carotene	8.5	8.4
IIA	hydroxy-alpha-carotene	0.5	0.15
	cryptoxanthin	10.0	3.1
IIB	P352	—	0.15
	cryptoxanthin 5,6,5′,6′-diepoxide	0.2	0.5
	hydroxy-alpha-carotene 5,6-epoxide	—	0.10
	cryptoxanthin 5,6-epoxide	0.4	0.7
	hydroxy-alpha-carotene 5,8-epoxide	—	0.09
	cryptoxanthin 5,6,5′,8′-diepoxide	—	0.04
	Ψ-cryptoflavin	0.4	4.7
	P399	—	0.26
IIIA	lutein	1.3	0.6
	zeaxanthin	1.5	0.8
	reticulataxanthin	—	0.26
IIIB	antheraxanthin	11.6	3.1
	flavoxanthin-like	—	0.16
	mutatoxanthins	0.5	0.6
IIIC	violaxanthin	45.6	26.4
	luteoxanthins	2.5	21.5
	auroxanthins	—	0.3
IV	valenciaxanthin	2.2	1.3
	sinensiaxanthin	2.2	2.7
	trans-neoxanthin	0.5	—
	valenciachrome	0.3	0.9
	trolliflor-like	0.4	—
	trollixanthin-like	1.2	0.8
	sinensiaxanthin-like	0.3	1.8
	trollichrome-like	0.17	—

[a] See Table 1 for explanation of I, IIA, IIB, etc.
[b] See Table 3 for explanation of terms P418, etc.

absorption maxima of the lower band were practically unchanged, whereas those of the upper band changed from 473, 444, and 422 mμ to 449, 426, and 403. This indicates that the lower band is a hydroxy-α-carotene, the upper cryptoxanthin 5,6-(or 5′,6′-) epoxide. A fraction similar to the latter obtained from Meyer lemon peel (Curl, unpublished) had an N_{100} of 48, hence would be expected to occur in part in fraction IIA ($N_{100} = 56$; IIB = 39).

In fraction IIB of the peel carotenoids, the principal constituent had a spectral absorption curve resembling that of cryptoflavin (or a cis-isomer), with absorption maxima in petroleum ether at 450, 424, and 403 mμ, corresponding to a conjugated system of 9 double bonds, with one end in a beta-ionone ring. This fraction did not give a positive test for epoxides in the hydrochloric acid–ether–methanol test; however, some 5,8-epoxides such as chrysanthemaxanthin (Karrer and Jucker, 1944) have been reported to give a negative hydrochloric acid–ether test. The N_{100} value of this substance in system I was 39, the same as that of fraction IIB. The considerable difference in this value from that of cryptoxanthin (56) indicates the presence of an additional hydrophilic group, but not a second hydroxyl group, since the N_{100} value of lutein (3,3′-dihydroxy-alpha-carotene) was 10. Lutein monomethyl ether (Curl, 1956) had an N_{100} value of 38 in this system, in good agreement with that of the cryptoflavin-like pigment, indicating that the latter may contain a hydroxyl and an ether or epoxide group at opposite ends of the molecule. If this substance were a 5,8-epoxide, it would be expected that it would be accompanied by a considerably greater quantity of the corresponding 5,6-epoxide isomer. No such substance was found. It appears that this substance is not cryptoflavin, hence it is referred to here as ψ-cryptoflavin (ψ for pseudo).

Mono- and diepoxides of cryptoxanthin were obtained from Meyer lemon peel (Curl, unpublished). A monoepoxide, which might be either the 5,6- or 5′,6′-epoxide, had an N_{100} value in system I of 48; the corresponding 5,8-(or 5′,8′-)epoxide (cryptoflavin?) had a value of 50. The 5,6,5′,6′-diepoxide had an N_{100} of 32. The N_{100} of ψ-cryptoflavin was about halfway between those of the monoepoxide and diepoxide of cryptoxanthin obtained from Meyer lemons.

Table 3. Spectral absorption maxima and N_{100} values in system I of some monol constituents obtained from navel orange peel.

Constituent	Spectral absorption maxima (mμ) in petroleum ether	N_{100} in system I
P352[a]	352, 337, 324	—
Cryptoxanthin 5,6,5′,6′-diepoxide	469, 439, 415	33
Hydroxy-alpha-carotene 5,6-epoxide	470, 441, 417	—
Cryptoxanthin 5,6-epoxide	473, 444, (422)	48
Hydroxy-alpha-carotene 5,8-epoxide	446, 419, 396	—
cis-cryptoxanthin 5,6,5′,8′-diepoxide	445, 418, 397	32
cis-Ψ-cryptoflavin	445, 418, 397	—
Ψ-cryptoflavin	450, 424, 403	39
P399	399, 377, 357	—

[a] P352 refers to substance with highest wavelength spectral absorption maximum at 352 mμ.

Several minor bands accompanied the main ψ-cryptoflavin band. These are listed in Table 3, with their spectral absorption maxima, and in several cases the N_{100} value also. Countercurrent distribution runs were carried out on several of the stronger minor bands in order to determine the N_{100} value as an aid in identification.

P352 (Tables 2, 3) had a principal absorption maximum at 337 mμ, with minor maxima at 352 and 324. The curve did not resemble that of any known carotenoid. This substance may be a terpene derivative occurring in the essential oil.

The lowest significant colored band was identified as cryptoxanthin 5,6,5′,6′-diepoxide. The spectral absorption curve was similar to that of violaxanthin, which is zeaxanthin 5,6,5′,6′-diepoxide. The N_{100} value in system I of this band was 33; it gave a greenish-blue color quickly in the hydrochloric acid–ether–methanol test. The countercurrent distribution curve had a pronounced hump with an N_{100} of 48. The tubes composing this hump were combined,

and had a spectral absorption curve like that of cryptoxanthin 5,6-(or 5′,6′-)epoxide.

The spectral absorption curve of the next band indicated it to be a mixture, so it was rechromatographed on lime, resulting in the separation of 4 well-defined bands, which were identified tentatively as a cis-cryptoxanthin 5,6,5′6′-diepoxide, hydroxy-alpha-carotene 5,6-(or 5′,6′)epoxide, cryptoxanthin 5,6-(or 5′,6′)epoxide and hydroxy-alpha-carotene 5,8-(or 5′,8′-)epoxide. The first band gave a greenish-blue color in the hydrochloric acid–ether–methanol test, the next two gave a green color, and the last was only slightly greenish. The last had much fine structure in the spectral absorption curve, indicating a conjugated double-bond system entirely in the central chain.

The next band was rather minor and was separated by a second countercurrent distribution into 2 substances, one of which was identified as a cis-isomer of cryptoxanthin 5,6,5′8′-(or 5′,6′,5,8-)diepoxide. It had a spectral absorption curve much like that of the "hydroxy-alpha-carotene" 5,8-epoxide, but had an N_{100} of 31 and gave a greenish-blue color in the hydrochloric acid–ether–methanol test.

The next band above was just below and not separated from the principal band. The spectral absorption curve indicated it to be a cis-ψ-cryptoflavin. This gave a negative hydrochloric acid–ether–methanol test, as did ψ-cryptoflavin.

There was some color, but no well-defined bands above ψ-cryptoflavin on the column. The eluate had pronounced maxima at 399, 377, and 346 mμ in petroleum ether, indicating the presence of a substance (P399) with about 6 conjugated double bonds.

Chromatography of fraction IIIA. Chromatography of the diol fraction showed zeaxanthin and lutein in rather small amounts. The peel carotenoids contained also a smaller red band above zeaxanthin, with one rather broad maximum in benzene at 474 mμ, with an inflection at *ca.* 508. This is apparently the same substance previously found in small amounts in Valencia peel carotenoids (Curl and Bailey, 1956) and in much greater quantity in tangerine peel (Curl and Bailey, 1957a). This fraction was referred to in earlier publications as being capsanthin-like (Curl and Bailey, 1956) or hydroxy-canthaxanthin-like (Curl and Bailey, 1957a), but more recent work (unpublished) has shown it to be neither of these: capsanthin had a much lower N_{100} value in system II, while the spectral absorption curve of canthaxanthin in hexane was quite different. The substance occurring in orange and tangerine peels has been named reticulataxanthin.

Chromatography of fraction IIIB. In both pulp and peel, the 5,6-epoxide, antheraxanthin, was found in much greater amount than the isomeric 5,8-epoxides, the mutatoxanthins. The fruit used was ripe but firm, and there was apparently little contact of the acid with the carotenoids in the pulp.

Chromatography of fraction IIIC. Here also, and especially in the pulp, the 5,6,5′,6′-diepoxide violaxanthin occurred in greater amount than the 5,8-epoxide isomers. Very little auroxanthins, the 5,8,5′,8′-diepoxide isomers, were found in the peel extract, and none in the pulp.

Chromatography of fraction IV. Both peel and pulp extracts contained small amounts of valenciaxanthin and sinensiaxanthin, two 5,6-epoxide polyols of incompletely known structure (Curl and Bailey, 1954). In the pulp carotenoids, the lowest, well-defined orange band (spectral absorption maxima in benzene 482, 451, 426 mμ) was identified as trans-neoxanthin by its behavior on countercurrent distribution in system III, where the N_{100} value was 60, in good agreement with that of neoxanthin from leaves. Neoxanthin has not been found in Valencia oranges. The next band above trans-neoxanthin had identical spectral absorption maxima in benzene, but on countercurrent distribution in system III the N_{100} value was 28; the distribution curve showed minor impurities at N_{100} of about 46 and 55. This great difference in N_{100} values probably indicates the presence of an extra hydroxyl group. Because neoxanthin is apparently a triol monoepoxide, the second band is probably a tetraol monoepoxide, perhaps trolliflor, $C_{40}H_{56}O_5$ (Lippert and Karrer, 1956). Above this band on the column were a number of other minor bands not well separated, including two that had a

green fluorescence in ultraviolet light, apparently valenciachromes a and b. The fractions eluted were mixtures as judged by the spectrophotometric curves; the maxima indicated the presence of a cis-trolliflor (or cis-trollixanthin)-like substance or substances, a sinensiaxanthin-like substance, and a trollichrome-like substance (a 5,8-epoxide). Because of the complexity of the mixture and the small amount present, it was not investigated further. The situation was similar with the peel carotenoids in the bands above sinensiaxanthin. Here also, there was another substance with spectral absorption maxima similar to those of sinensiaxanthin, but higher on the column.

It was noted earlier, in work on the carotenoids from the peels of Valencia oranges (Curl and Bailey, 1956), Ruby Red grapefruit (Curl and Bailey, 1957b), and tangerines (Curl and Bailey, 1957a), that several of the components of the diepoxide diol and polyol fractions contained impurities with very high single maxima in the region of 300 to about 335 mμ (in benzene). Similar maxima were present in some of the fractions from navel orange peel. It appears that these maxima are attributable to completely methylated flavanones; the substance accompanying violaxanthin has been tentatively identified as nobiletin by comparison of the ultraviolet and infrared absorption spectra.

Absorption maxima: in benzene, 324 mμ; in 95% ethanol, 328, 270 and 250 mμ; infrared (Beckman IR-5), principal maxima at 3.41, 6.12, 6.23, 6.60, 6.83, 7.09, 7.32, 7.49, 7.86, 8.19, 8.46, 8.70, 8.97, 9.24, 11.93 mμ (comparison made with a sample of nobiletin obtained by Roy Teranishi of this laboratory, from oil of orange). Swift (1960) recently isolated nobiletin from Valencia orange peel.

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Oxidative Damage to Amino Acids, Peptides and Proteins by Radiation[a]

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SUMMARY

Oxidative damage to amino acids, peptides, and proteins by radiation was measured as total peroxides and thiobarbituric acid reactants. Two types of thiobarbituric acid reactants were formed in low yields.

In radiation sterilization of food, and also in radiation biology, peroxidation of constituents is an important mechanism of damage (Latarjet and Haissinsky, 1958; Ord and Stocken, 1956; U. S. Army Quartermaster Corps, 1957). Most previous research deals with the oxidative damage to lipids by ionizing radiations, since the lipids are the most labile components of foods and animal tissues. Peroxides and hydroperoxides are produced by irradiation of amino acids and proteins (Latarjet, 1956; Okada, 1958; Ord and Stocken, 1956) and these appear to be important in radiation damage.

Amino acids, when irradiated, give rise to a spectrum of products among which carbonyls and peroxides are formed by oxidative reactions (Duran and Tappel, 1958; Okada, 1958). The possibility of extensive deamination and decarbonylation reactions leading to the formation of short-chain aldehydes like malonaldehyde suggested use of the thiobarbituric acid (TBA) reaction for study of the oxidative changes in amino acids, peptides, and proteins. The TBA reaction has been widely used for assay of oxidative rancidity of lipids in various foods (Sinhuber and Yu, 1958; Sinhuber *et al.*, 1958; Tarladgis *et al.*, 1960). Smith *et al.* (1960) described the TBA reaction with whole irradiated beef.

The present paper reports a survey of total peroxides and TBA reactants produced by irradiation of amino acids, some peptides, and proteins.

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EXPERIMENTAL

Sample preparation and irradiation. The amino acids, peptides, and proteins were dissolved in deionized water, neutralized to pH 7, and made up to a 0.1% solution (0.01% cystine and 0.05% tyrosine). These were oxygenated (1 atm) in polyethylene bottles with large oxygen head space, and the bottles were sealed in No. 2 cans and transported at dry-ice temperature to and from the irradiation facility. Samples were also irradiated anaerobically under N_2.

The samples were irradiated at ambient temperature by exposure to 0.6–2 mev γ-radiation from spent radioactive fuel rods at the Materials Testing Reactor, Idaho. A suitable dose of 5×10^6 rad was selected for the general survey; however, a fairly broad range of doses was given in the case of methionine, glutathione, and proteins to obtain a general pattern. The samples were immediately frozen after irradiation and stored at −20°C until analyzed.

Analysis. For the thiobarbituric acid reaction, 1 ml of 0.75% TBA in 0.1*N* HCl was mixed with 4 ml of the sample and heated 15 min at 100°C. Absorbance spectra were determined with a Beckman DK-2 spectrophotometer. Malonaldehyde concentration was calculated, using E_M at 530 mμ as 1.56×10^5 (Sinhuber and Yu, 1958).

Total peroxides were determined by the iodometric method. A suitable aliquot (5–10 ml) of the sample was gassed with pure nitrogen for 5 min while being mixed with an equal volume of glacial acetic acid, and excess of KI crystals were added. The iodine liberated was titrated by sodium thiosulfate (.005*N*), using starch as the internal indicator.

RESULTS AND DISCUSSION

It is evident that under the conditions of these experiments, we measure only the relatively stable peroxides and other oxidative products formed by irradiation. Table 1

Table 1. Peroxides and thiobarbituric acid reactants in irradiated solutions of amino acids and peptides.

Amino acid or peptide	G value for peroxide, molecules of peroxide per ion pair	TBA reactants: Millimole malonaldehyde per mole amino acid	TBA reactants: Absorbance 448 mμ per mole amino acid
Hydroxyproline	0.86	0.00	0
Arginine	0.79	4.1	0
Proline	0.67	0.36	41
Valine	0.55	0.07	14
Alanine	0.53	0.00	0
Glutamic acid	0.53	4.3	0
Phenylalanine	0.48	0.62	105
Threonine	0.43	0.09	20
Leucine	0.41	0.04	7.9
Lysine	0.34	0.05	7.7 (445 mμ)
Glycine	0.28	0.006	3.2
Homocystine	0.26	1.14	0
Serine	0.26	0.45	95
Tryptophan	0.22	0.00	47
Tyrosine	0.21	0.13	37 (440 mμ)
Aspartic acid	0.15	0.10	12.8
Methionine	0.03	1.44	0
Histidine	0.03	0.00	0
Cysteine	0.00	0.15	42
Glycyl-methionine	0.03	0.08	0
Glycyl-tyrosine	0.08	0.09	46 (440 mμ)
Glutathione	0.1	0.00	1.1
Methionine sulfone	0.04	0.25	0
Methionine sulfoxide	0.07	0.71	0

lists the results obtained with irradiated (O_2, 5×10^5 rad) amino acids, and some peptides, in terms of total peroxides and TBA reactants.

These results show that the formation of peroxides and TBA reactants like malonaldehyde is a general reaction, though the amounts of products formed differ considerably. In fact, the values presented here correspond to only one dose (5×10^5 rad) and may not be the maximum for these components. This is brought out by the data in Table 2, where a typical amino acid like methionine is subjected to varying doses of ionizing radiation under oxygen or nitrogen. As would be expected, under anaerobic conditions very little peroxides and carbonyls are detected. Also, the peroxidative damage increases with increasing dose, except that, at very high doses of 10^6 and 10^7 rad there is a significant decrease in their concentration, suggesting a further breakdown of the oxidative products of irradiation. The yield of total peroxides in this study (Table 1) is lower than that obtained by Okada (1958), who used conditions favoring maximum yield.

Calculations of yields as G values showed that the TBA reactants are minor products of irradiation. Among the amino acids, ar-

Table 2. Peroxides and thiobarbituric acid reactants in irradiated 0.1% methionine.

Dose (rad × 10^{-5})	Gas	G value for peroxide (molecules peroxide / ion pair)	TBA reactants millimole (malonaldehyde / mole amino acid)
.01	O_2	0.00	0.00
.1	O_2	0.09	0.03
1.0	O_2	0.03	0.30
5.0	O_2	0.03	1.44
10	O_2	0.02	0.78
100	O_2	0.00	0.83
1.0	N_2	0.00	0.057
5.0	N_2	0.00	0.086
100	N_2	0.03	0.40

ginine, glutamic acid, methionine, and homocystine gave the highest yields of malonaldehyde. No peak corresponding to malonaldehyde was observed in the case of hydroxyproline, alanine, tryptophan, and histidine. In comparison with methionine, glycylmethionine formed very little malonaldehyde and methionine sulfoxide and methionine sulfone gave considerably reduced amounts.

Mechanisms for the formation of malonaldehyde in the radiation breakdown of methionine include the following:

scission ↓ (at S–C); H; decarboxylation ↓ (at C–COOH)

$CH_3{-}S{-}CH_2{-}CH_2{-}C(H)(NH_2){-}COOH$ ← oxidative deamination (at NH_2)

↓

$[-CH_2-CH_2-CHO] \xrightarrow{\text{oxidation}} OHC-CH_2-CHO$ (malonaldehyde)

Comparable yields of malonaldehyde from irradiated homocystine support such a mechanism. Homocystine would be expected to undergo –S–C– scission, decarboxylation, and oxidative deamination by similar mechanisms and at similar levels as methionine. Known mechanisms of radiation damage to amino acids that would give malonaldehyde from glutamic acid are:

decarboxylation ↓ (at HOOC–C); H; decarboxylation ↓ (at C–COOH)

$HOOC{-}CH_2{-}CH_2{-}C(H)(NH_2){-}COOH$ ← oxidative deamination (at NH_2)

↓

$[-CH_2-CH_2-CHO] \xrightarrow{\text{oxidation}} OHC-CH_2-CHO$ (malonaldehyde)

Besides the TBA reactant with maximum absorbance at 530 mμ, which is characteristic of malonaldehyde, some amino acids gave a TBA chromophore with maximum absorbance in the 448 mμ region. Since the absorbance of this 448 mμ chromophore was, on the average, as large as the 530-mμ malonaldehyde absorbance, the values are included in Table 1. A number of carbonyl compounds, particularly aromatic aldehydes, are known to give TBA reactants that absorb in the 450 mμ region. In a recent article, Patton (1960) described the yellow pigments (456 mμ) formed in a TBA reaction by epihydrin aldehyde and glyceraldehyde. It would be of interest to identify unknown TBA reactants formed in irradiation of amino acids and peptides, but the yield is very low.

Extension of the TBA reaction to irradiated pure proteins, cytochrome *c* and hemoglobin, gave a more complex picture, with formation of chromophores having characteristic absorption in the region 448 mμ to 465 mμ, and shoulders at 493 mμ and 612 mμ. The yields of these unidentified TBA reactants were quite low. TBA reacts with a variety of carbonyls to give rise to a spectrum of products absorbing between 420 mμ and 540 mμ. In the case of most of these protein derivatives, however, a fairly strong absorption in the region 440–449 mμ was observed after reaction with TBA. Glutathione gave a characteristic double peak at about 424 mμ and 448 mμ, and at the highest dose tested (5×10^7 rad, O_2) showed additional absorption at 530 mμ, indicating malonaldehyde formation.

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An Olfactometer for Rapid and Critical Odor Measurement

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SUMMARY

An olfactometer is described that will deliver calculated amounts of odor to subjects in a rapid and reproducible manner. It was found that sparging air through a test liquid will saturate the air at the vapor pressure of the liquid. Sensitivities, for a panel, were determined at two levels of acetic acid concentrations; they were 0.939 deviates per mg ($\times 10^{-5}$) per L for a concentration of 1.90×10^{-6} mg per L, and 0.587 deviates per mg ($\times 10^{-5}$) per L for a concentration of 3.37×10^{-5} mg per L. Some modifications of the olfactometer are suggested and discussed.

Testing olfactory sensitivity is tedious and subject to numerous errors, and offers no standardized methods such as are available in taste studies. Many different types of studies with a variety of equipment have been made at one time or another (Deininger and McKinley, 1954; Elsberg and Levy, 1956; Schneider and Wolf, 1955; Wenzel, 1948). Of all the tests, perhaps the most popular have been the Elsberg blast injection test and the sniffing test. Since introduction of the Elsberg-Levy blast injection technique (Elsberg and Levy, 1956), much work has been devoted to the use of this instrument for olfactory measurements with humans. In 1953, however, Jones provided empirical evidence against use of this instrument for threshold determinations. It was concluded that thresholds obtained by the blast injection technique were not directly comparable to those obtained by another method, the sniff test. Further elaboration by Jones (1955) demonstrated this sniff test as a reliable means for comparison of odorous materials. Wenzel (1955), working on similar problems, also discontinued use of the blast injection technique. Unreliable performances of trained subjects and "artificiality of the situation," which raised the question of relating this to normal breathing, were major reasons listed. In its place Wenzel presented a method for utilizing natural breathing in an odor-free "environment." Our study, utilizing a similar apparatus, is described below in greater detail. With this olfactometer, no artificial device is needed to carry the odorous air to the olfactory region. Further, the uncontrollability and tedium of solution preparation have been eliminated in the design and construction of this apparatus. Also, it is possible to determine the concentration of odorous material in the vapor state, given the vapor pressure of the compound in question and conditions under which the test was performed.

In conjunction with the description of the olfactometer and the technique used, the results of testing a small panel for sensitivity to acetic acid at two odor concentrations are presented.

APPARATUS AND METHOD

Equipment. The design of the olfactometer (Fig. 1) is based on the earlier and cruder device reported by Mrak *et al.* (1959). In principle, the olfactometer is the same as that described by Wenzel (1955), but the design differs in several features. Both olfactometers are based on the premise that air may be saturated to a constant amount by contact with the test substance. The Wenzel apparatus used a static arrangement, where the air is first saturated in a large container and small amounts are diluted in a main air stream to arrive at the desired concentrations. With the ap-

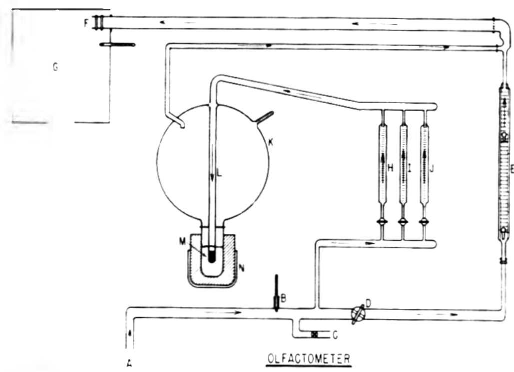

Fig. 1. Schematic. A) Incoming conditioned air; B) thermometer; C) air by-pass valve; D) control valve for large air flow; E) large rotometer (14 cu ft per min capacity); F) muffler; G) odor hood (Plexiglas); H) I) J) small rotometers; K) diffusion bulb (5 L); L) small air-flow delivery tube; M) sintered-glass sparger (coarse); N) water bath (control of test liquid temperature).

paratus described here, it is assumed that air passed through the test substances in the proper manner will be saturated and hence may be delivered continuously for dilution in a main air stream.

Air is supplied by a Nash Compressor (Model MD571) at 21 CFM. The air pump was sealed with 42°F water with a secondary purpose of controlling the compressed-air temperature. The charcoal-filtered air is passed by appropriate valves and lines into the olfactometer. A portion of the air is by-passed through one (or more) of three flow meters (Fisher-Porter Nos. 2F-¼-20-5/35, 02F-⅛-16-5/35, and 08F-1/16-16-4/35; 0–8030 cc per minute) to the test liquid in the diffusion bulb. The bulk of the air is passed through a large flow meter (Fisher-Porter 14 CFM capacity) to a Plexiglas hood. The air metered into the diffusion bulb is saturated by passing through a sintered-glass sparger immersed in the test liquid. The saturated air is delivered back to the main air stream.

Calibration of equipment. Adequate correction curves for pressure and temperature variations are furnished with the meters and are used to adjust indicated flows to absolute values. To determine the actual amounts of acetic acid delivered to the odor hood, saturated air (at varying flow rates) was trapped and measured in solutions of 0.01N sodium hydroxide. The results of this calibration (Table 1) indicate the method is satisfactory and the deviations from the calculated amount are due to expected errors in the calibration routine.

True flow rates (Table 2) were determined at the actual working pressures at the various set points. Because of the critical region used on the middle-size flow meter and a method error in

Table 1. Determination of completeness of air saturation with acetic acid.

Flow rate (cc min)	Calculated amount (mg min)	Determined amount (mg/min)
400	21.2	22
600	31.7	29
670	35.3	38
977	51.4	55
1770	92.5	103
2000	107	132
3430	187	198
4370	246	247
5300	301	300
7300	411	400
8530	480	480

reading this meter (tending to read the value low 0.1 or 0.2 set points) at the set point 4.5, this value was deleted from the calculations.

Calculation of amount of odor delivered. The vapor pressure of the test substance may be used to calculate the amount of odor in the saturated air. The calculation is represented by the formula:

$$\frac{MW \times Vp \times T_{std}}{R \times P_{std} \times T} \quad \text{(amount in g/L or mg per ml)}$$

MW = molecular weight of test compound
Vp = vapor pressure in mm of test compound at the operating temperature
T_{std} = temperature in degrees Kelvin at standard conditions
R = volume occupied by one molecular wt of gas at standard conditions (22.4 L)
P_{std} = pressure at standard conditions in mm
T = operating temperature in degrees Kelvin

To convert to mg per minute, multiply flow rate times saturation concentrations. mg per cc × cc per min = mg per min

If the pressure is appreciable a suitable correction may also be included in the formula. The odor-saturated air diluted by the main air stream is calculated in mg per L.

$$\frac{\text{flow rate of odor-saturated air}}{\text{flow rate of main air stream}} = \frac{\text{mg per min}}{\text{L per min}} = \text{mg per L}$$

The volume consideration of the small flows may be ignored since it is too minor to cause significant change in total flow.

Panel. A group of 8 men and 4 women 22–55 years old were selected from the department staff. Only one of the panel had had experience with this type of testing.

Design. Individual sensitivities at two levels of acetic acid (1.90×10^{-5} and 3.37×10^{-6} mg per L)

Table 2. Calibration of flow meters under actual experimental operating conditions.

Set points	Delivery rate (ml/min)
Small-size meter	
5.2	14.7
6.3	28, 26
8.1	61
9.8	112, 119, 115
10.6	128, 127, 130
11.4	149, 147
12.7	189, 189
14.0	222, 238
15.2	263, 271
15.5	286, 286
Middle-size meter	
4.3	230, 219, 240
4.4	245, 254, 240
4.5	288, 286, 274, 277
5.1	345, 358, 345

were determined. Three concentrations on either side of each midpoint were 0.20, 0.87, 1.57, 2.20, 2.94, and 3.89×10^{-5} mg per L, and for the higher midpoint 0.37, 1.74, 2.82, 3.80, and 4.94×10^{-5} mg per L. The constant-stimuli method of presentation was used. In no paired comparison were "no difference" responses allowed. Six randomized pairs were presented to each subject each day for 8 days. Four replicas of each pair were given to each subject. A definite testing time was assigned to each panel member. The question asked was: Which stimulus level (of a presented pair) has the more intense acetic acid odor? Each sample was presented for 10 sec, followed by 10 sec of no stimulus to allow removal of the odor.

Statistical calculations. The method of maximum likelihood is an efficient statistical procedure for analyzing data obtained by the constant-stimuli method. It yields the equation of a regression line with the corresponding confidence band and the standard errors of estimate of the deviates and of the regression coefficient. The procedure is fully explained by Jones (1957). By use of weighted deviates the method adjusts for discrepancies between the normal and binomial distributions when the percentage of responses is very high or very low; hence 0 or 100% responses may be included in the calculations. The data are plotted on normal-probability paper with percentage of responses termed high, being plotted against stimulus concentrations, and a provisional regression line is formulated, on the basis of which a survey analysis may be applied to the data using χ^2 to check for fit and homogeneity. In this analysis, responses below 10% and above 90% should not be included. The procedure furnishes an indication of the adequacy of the provisional line and the sufficiency of the data.

The method of measuring flavor intensity as developed by Litchfield and Wilcoxon (1949) and presented by Sinsheimer (1959) is not applicable to these odor measurements, since, with these data, response does not follow a logarithmic relationship.

Adjusted χ^2 may be used to analyze differences in the number of incorrect responses when comparing subjects by days.

EXPERIMENTAL RESULTS

The number of incorrect responses on a daily basis for the two measurements are summarized (Table 3). χ^2 analysis shows no significant difference between subjects.

Table 3. Number of incorrect responses on a daily basis by 12 subjects.

Days	1	2	3	4	5	6	7	8	9	10	11	12	Total (daily)	Total (complete sets)
1	0	1	2	1	0	3	2	3	0	1	2	0	15	27
2	1	3	0	0	0	2	1	1	1	1	1	1	12	
3	1	2	3	1	1	1	1	2	0	1	1	1	15	24
4	1	1	1	1	1	0	0	0	1	1	0	2	9	
5	2	0	1	0	0	1	2	2	2	2	1	3	16	27
6	0	2	1	1	1	0	1	3	0	0	1	1	11	
7	1	0	0	1	2	0	1	2	1	1	0	1	10	24
8	1	0	1	1	2	2	1	1	2	0	0	3	14	
Total	7	8	9	6	7	9	9	14	7	7	6	12	102	
N	44	44	44	44	44	44	44	44	44	44	44	44	528	

To determine if 6 incorrect responses are significantly different from 14: χ^2 (adj.) = 3.18 (not significant at 5% level).

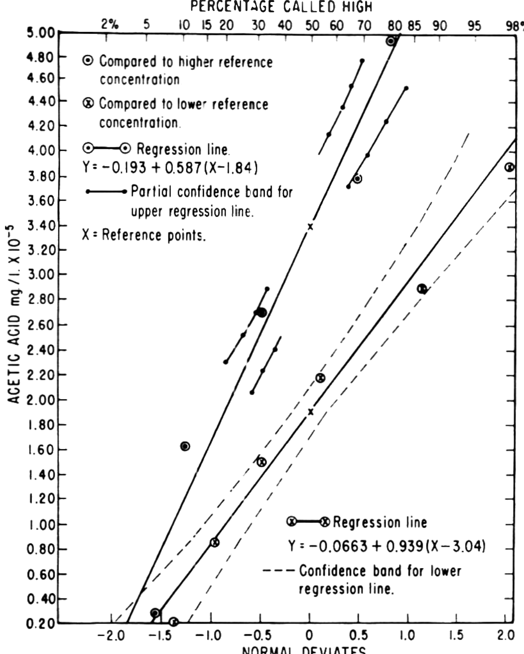

Fig. 2. Points and regression lines with confidence bands for panel sensitivity at two acetic acid levels.

The percentage of responses called more intense versus the acetic acid concentrations (in the hood) is plotted on normal-probability paper (Fig. 2). Provisional regression lines drawn were found to fit reasonably well (Table 4). Since homogeneity of data was indicated, the method of maximum likelihood was used in the analysis. For the lower (Table 5) and the higher (Table 6) reference points, regression lines, standard errors of estimate, and confidence bands were determined.

If the analysis of regression had resulted in a significant residual term, then determinations of the standard errors of estimate and confidence bands would have to be based on sums of squares of this χ^2 term. In both instances the regression analysis for the residual terms were not significant. The confidence bands are indicated on Fig. 2.

It should be stated that in analyses of this type each pair of stimuli should be presented to each subject only once if the results are to be applied to populations. This work is presented to indicate primarily the usefulness of the olfactometer, and the results may be interpreted to indicate the sensitivity of the panel only. Since no significant differences were found for the individual panel members it may be assumed that their individual error terms are normally distributed, which is a main criterion of this analysis.

Table 4. Provisional χ^2 analyses of data and fit.

Source of χ^2	df	χ^2	Probability
Higher mid point (3.37×10^{-5} mg/L)			
Total χ^2	3	0.824	
Pooled χ^2	1	0.011	
χ_h^2	3	0.813	$.90 > p > .80$
Lower midpoint (1.91×10^{-5} mg/L)			
Total χ^2	4	0.881	
Pooled χ^2	1	0.048	
χ_h^2	4	0.833	$.95 > p > .90$

Table 5. Maximum likelihood solution using lower reference point: regression line $Y = -0.0663 + 0.939\ (X - 1.84)$, regression coefficient $b = 0.939$.

Analysis of regression				
Source of χ^2	df	Sums of squares	χ^2	Probability
Regression	1	1.993	95.90	
Residual	4	0.118	5.66	$.30 > p > .20$
Total	5	2.111		

SE(y) = 0.091
SE(b) = 0.096
95% confidence band for Y
$Y' = \pm 1.96 \sqrt{0.00837 + 0.00921\,(X + 0.070)^2}$

Table 6. Maximum likelihood solution using higher reference point: regression line: $Y = -0.193 + 0.587\ (X - 3.04)$, regression coefficient $b = 0.587$.

Regression analysis				
Source of χ^2	df	Sums of squares	χ^2	Probability
Regression	1	1.502	72.10	
Residual	3	0.042	2.02	$.70 > p > .50$
Total	4	1.544		

SE(y) = 0.0945
SE(b) = 0.0692
95% confidence band for Y
$Y' = \pm 1.96 \sqrt{0.008934\,(X + 0.326)^2 (0.00479)}$

The regression coefficients are of major interest since they are an efficient tool for further psychological and physiological investigations. The regression coefficients in these measurements indicate a greater sensitivity of the panel when tested at the lower concentrations [0.939 deviates per mg ($\times 10^{-5}$) per L] as compared to the higher concentrations [0.587 deviates per mg ($\times 10^{-5}$) per L].

DISCUSSION

Slight trigeminal effects on individuals at the higher acetic acid concentrations (14 mg per min into main stream) did not appear to affect the results measurably.

One disadvantage of the present apparatus is that there is no fine temperature control of the air entering the hood. Air temperatures in the hood varied between 74°F (first day) and 92°F (last day). Insufficient tests were done to determine effects on the panel. To remedy this temperature variation a control is being installed in the main air stream, consisting of a chilling coil followed by a controlling heater. Temperatures may then be regulated closely, regardless of intake air temperatures.

The possibility of using this olfactometer to measure sensitivities to mixtures is conceivable. A modification of the sample holder (Fig. 3) would, by allowing liquid mixtures to flow through the holder continuously, make this feasible by maintaining a given concentration at the sparging area.

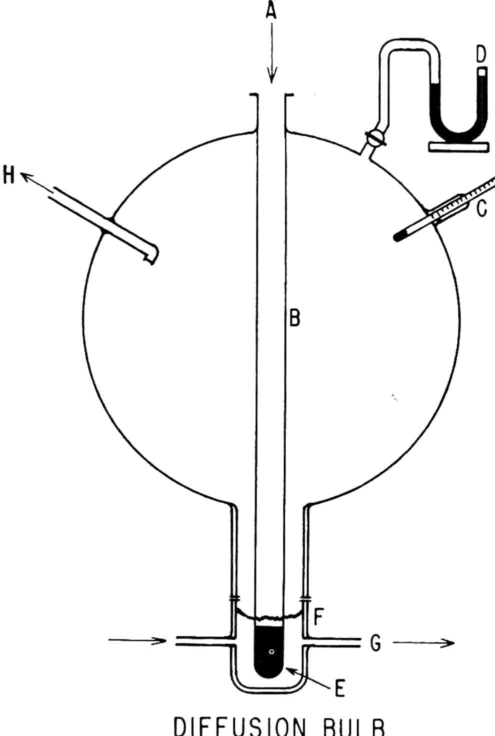

Fig. 3. Schematic. A) Incoming small air flow (from small rotometers); B) 3-L bulb; C) thermometer; D) manometer; E) coarse sintered-glass sparger; F) removable portion of bulb (liquid holder); G) inlet and outlet for mixtures being tested; H) outlet for saturated air.

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