Journal of
FOOD SCIENCE

SUBSCRIPTION RATES

To members of the Institute of Food Technologists, $7.50 per volume.

Non-Member Subscription Rates: Domestic, $8.50 per year: Canada, Mexico, Central and South America $10.00 per year plus $1.00 postage, all other Countries $10.00 per year plus $2.00 postage; Single copies, Domestic, $1.50 each; Foreign, $1.75 each.

All subscriptions are entered to begin with the first issue of the current volume. One volume per year published starting with the January-February issue. Subscription orders received after February 15 will be pro-rated for the balance of the current volume. Issues published prior to date of receipt of subscription order will be figured at the single copy price if available.

Copies of many back numbers are available at the single copy rate. Remittance to accompany order.

CORRESPONDENCE

Editorial: DR. G. F. STEWART, Executive Editor, P. O. Box 164, Davis, California.

Non-Member Subscriptions, Back Volumes, Single Copies, and Reprints: FOOD SCIENCE, 510-522 N. Hickory Street, Champaign, Ill.

Member Subscriptions: THE INSTITUTE OF FOOD TECHNOLOGISTS, 176 W. Adams St., Chicago 3, Ill.

Advertising: CALVERT L. WILLEY, Business Manager, 176 W. Adams St., Chicago, Ill.

CLAIMS

Claims for copies lost in the mails must be received within 30 days (90 days foreign) of the date of issue. Notice of change of address must be received two weeks before date of issue.

NOTE

The Institute of Food Technologists assumes no responsibility for statements in articles appearing in FOOD SCIENCE. The opinions expressed do not necessarily reflect policies of the Institute of Food Technologists.

Manuscripts for FOOD SCIENCE should conform to the style used in the journal and be submitted in duplicate to the Executive Editor. Authors of papers intended for FOOD SCIENCE should request from the Editorial office a copy of the booklet "Research papers for IFT journals." FOOD SCIENCE reserves the privilege of editing manuscripts to make them conform with the adopted style of the journal or returning them to the author for revision. Changes made by the Editorial Office may be reviewed by authors when they examine proof.

Published by the Institute of Food Technologists six times a year. Printed by The Garrard Press, 510-522 N. Hickory Street, Champaign, Illinois. Entered as second-class matter at the Post Office at Champaign, Illinois, under the act of March 3, 1879. Accepted for mailing at special rate of postage provided for in amended Section 34.40(e), pursuant to the provisions of Public Law 233, approved October 8, 1951.

MARCH-APRIL, 1962 **Volume 27, Number 2**

An Official Publication of the Institute of Food Technologists

Journal of FOOD SCIENCE

MARCH-APRIL, 1962 Volume 27, Number 2

CONTENTS

Kinetic Studies on the Heat Inactivation of Peroxidase in Sweet Corn[a]

H. Y. YAMAMOTO,[b] M. P. STEINBERG, AND A. I. NELSON
Department of Food Technology, University of Illinois, Urbana, Illinois

(Manuscript received August 7, 1961)

SUMMARY

A kinetic procedure employing D values was used instead of the usual end-point method to study heat inactivation of peroxidase in whole-kernel sweet corn. Results at 150–200°F indicated that a heat-labile fraction and a heat-stable fraction were being inactivated. The resistant fraction represented 5% of total enzyme activity, and was concentrated in the pericarp. Increasing the blanch time at 200°F from 2 to 5 min decreased residual enzyme activity from 3.3% to 1.7%.

Inactivation of the heat-resistant fraction at 210–290°F also followed a first-order reaction. The phantom inactivation-time curve showed that an HTST process based on microbial destruction could leave residual enzyme activity.

In the conventional thermal process for canned foods, i.e., at retort temperatures of 250°F and below, enzymes are inactivated in some time less than that required for achieving commercial sterility. The situation may be the reverse at elevated retort temperatures. Vetter *et al.* (1958a, 1959), in their work on inactivation of sweet-corn peroxidase, showed that it is possible to find residual enzyme activity in whole-kernel corn given a high-temperature short-time process based on bacterial destruction. Their data indicated that the heat inactivation-time curve for peroxidase was biphasic, showing a smaller slope above a critical temperature.

The heat inactivation curve used by Vetter *et al.* (1958a, 1959) was based on an "end-point" determination. A series of samples were heated at a given temperature for various times and analyzed for residual enzyme activity. The enzyme was considered inactivated when the assay method (Vetter *et al.*, 1958b) could detect no activity and the heating time was corrected for heat penetration lag. This is the classical approach also used to obtain a thermal death-time curve for bacterial spores. The position of the resulting curve in either case depends on the sensitivity of the assay method.

The disadvantage of the end-point method was avoided in the present work by taking an alternative approach, as outlined by Esselen and Pflug (1954) and Stumbo (1949). In this, the *rate* of inactivation is determined at each of several temperatures. The plot of these data gives a "phantom" heat-inactivation curve, which is not fixed in space but can be defined by selecting a starting and a final concentration. Such a kinetic study was made on the inactivation of sweet-corn peroxidase in the temperature range of 150–290°F for this report. Although Farkas *et al.* (1956) used such an approach at temperatures to 212°F, this appears to be the first *rate* study on high-temperature inactivation of enzymes in vegetables.

EXPERIMENTAL

Materials. A 500-watt Precision Scientific Co. constant-temperature bath was used to obtain constant temperatures to ±0.1°F. Two auxiliary heating elements of 550 and 1000 watts activated by a magnetic switch were added to the original equipment.

For heating whole-kernel corn in water, cylindrical wire cages approx 2 in. in diameter and 3 in. high were constructed of ⅛-in. wire mesh. Thermal death-time (TDT) cans, 208 × 006, were

[a] M.S. thesis in Food Technology, by the senior author, University of Illinois, Urbana, Illinois.

[b] Present address: College of Tropical Agriculture. University of Hawaii, Honolulu 14, Hawaii.

used to heat whole-kernel corn in the oil bath. Each cage and can was attached to a handle about 18 in. long.

Unblanched whole-kernel Golden Cross sweet corn of optimum maturity at about 72 percent moisture content was used. The kernels were mechanically removed using a medium cut (about ¼-kernel length) and cleaned so tip cap and cob tissue were absent. This corn, which had been stored at −40°F in 30-lb tins, was used for the greater part of this study. The frozen kernels were thawed in a flat porcelain pan under a small fan for 1 hr at room temperature. They were washed several times with tap water and drained before use. Fresh corn, obtained from the local market, was also used. The kernels were removed from the cob with a knife and then treated as thawed whole-kernel corn.

Procedures for heating whole-kernel sweet corn. Depending upon the processing temperature, two procedures were used to heat the corn. Over the range of 170–200°F, approx 20-g samples of whole sweet-corn kernels were immersed in a soft-water bath at the proper temperature for 5–600 sec. The wire containers were agitated during heating and subsequent cooling in an ice-water bath. The cooled samples were drained for 3 min, sealed in "C" enameled 211 × 400 cans, and stored 72 hr at 34°F before analysis for peroxidase.

For treatment at 210–290°F, overlapping all temperatures used in canning, a mineral-oil bath was used and the heating times ranged from 60 to 600 sec. Eleven grams whole-kernel corn was sealed with 7.5 cc soft water in the thermal death-time cans. These small cans were agitated during the first 2 min of heating and while cooling in ice water. The cans were then stored 72 hr at 34°F. Finally, the samples were drained 3 min before enzyme analysis.

For temperatures from 210 to 245°F the indentation in the can bottom was hammered flat. This tended to reduce come-up time. Above these temperatures the can was not flattened, since the internal pressure buckled the can somewhat, giving the same result.

Heat penetration determinations. Heat penetration in whole-kernel corn held in the wire cages was determined by impaling a kernel on a copper-constantan thermocouple and following the temperature rise with a portable potentiometer.

A twelve-point Minneapolis-Honeywell recording potentiometer was used to study heat penetration into the TDT can. A bare-junction type of Ecklund thermocouple, attached to the TDT cans by a special fitting, was sealed in the can and the leads connected to all twelve points of the potentiometer. Each point, therefore, recorded the internal temperature of the can.

Peroxidase assays. Both the orthophenylenediamine (OPDA) and ascorbic acid methods for determination of peroxidase activity were used. The OPDA analysis was that of Vetter *et al.* (1958b), which had been specifically developed for determination of peroxidase in sweet corn with minor modifications (Yamamoto, 1958). It is based on the catalytic oxidation of OPDA by hydrogen peroxide with a colorimetric determination of reaction product. The enzyme activity was expressed in terms of optical density units (ODU) per gram of corn after 5 min of reaction.

The ascorbic acid method of analysis was carried out with the concentration of reagents recommended by Joslyn (1956, 1957) and according to the procedure detailed by Yamamoto (1958). It is based on the enzymatic oxidation of ascorbic acid by peroxide in the presence of 2,6-dichlorophenol indophenol. After 2 min reaction time the unoxidized ascorbic acid was determined by titration and the enzyme was calculated as mg ascorbic acid oxidized per gram corn.

Determination of inactivation rate. At each inactivation temperature, the residual enzyme activity remaining in the corn, expressed as percent of original (or zero heating time) activity, was plotted on a logarithmic scale against the heating time. Two points were read on each straight-line portion for calculation of the heat inactivation rate as defined by the "D" value, the time required to inactivate 90% of the enzyme present (Esselen and Pflug, 1954; Stumbo, 1949).

RESULTS AND DISCUSSION

The results are considered in two sections based on processing temperatures. The first covers the range 150–200°F, while the other is concerned with enzyme inactivation at 210–290°F.

Inactivation at 150–200°F. Fig. 1 plots the results of the experiment at 191°F as determined by OPDA analysis. This curve, showing percent of original enzyme activity remaining, on the logarithmic axis, after the various heating times, was typical of all those obtained in this temperature range. Identical data were obtained with fresh and frozen product, thus validating application of the results with frozen corn to processing of the fresh product. Also, when the ascorbic acid method was used to determine residual enzyme and the results were expressed and plotted in the same way, an almost identical curve (not shown) was obtained. This finding, shown later (Fig. 3), serves as a check

on the reliability of the procedures used and the results obtained.

Two resistances found. As expressed in Fig. 1, the heat inactivation curve consisted of an initial steep straight line, an intermediate curved portion, and a final straight line with a shallow slope. The same type of curve would be obtained if two microorganisms with radically different heat resistances were being destroyed at some lethal temperature (Ball and Olson, 1956). It was therefore concluded that two independent first-order inactivation reactions were taking place; the initial line represented inactivation of heat-labile enzyme, and the second, heat-resistant enzyme. The curved portion may be considered a transition zone.

There are several explanations for this finding. The peroxidase of sweet corn may be multiple in nature, with two enzymes or enzyme groups having two different heat stabilities and each being inactivated according to a first-order reaction. Multiplicity of peroxidase has been shown for horse-radish (Jermyn, 1952). Another possibility is that a protective mechanism such as formation of enzyme-substrate complex may be affecting the heat stability of a portion of the single enzyme. The initial rapid inactivation represents the disappearance of the unprotected peroxidase.

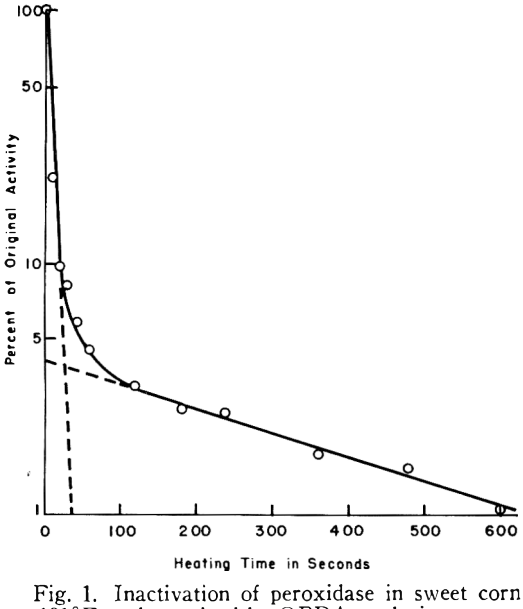


Fig. 1. Inactivation of peroxidase in sweet corn at 191°F as determined by OPDA analysis.

It would be of interest to know what portion of the total peroxidase activity exists in the heat-resistant form. This can be estimated by extrapolating the heat-resistant curve to zero time. As may be seen in Fig. 1, this level was approximately 4% of the total activity.

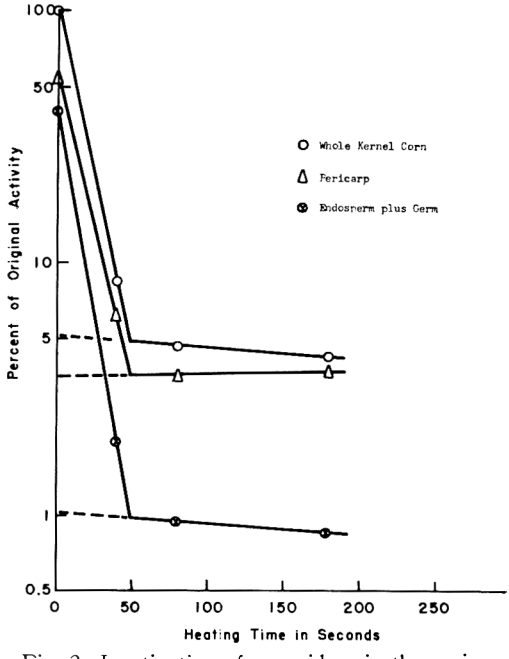


Fig. 2. Inactivation of peroxidase in the pericarp and in the endosperm plus germ fractions as compared to whole-kernel corn at 171°F.

Location of enzyme. It was noticed that particles of pericarp were deeply colored when the OPDA enzyme assay was employed. This could be due to a high concentration of enzyme in the pericarp. Therefore, an experiment was conducted to establish the location of the labile and resistant fractions in whole-kernel corn. Twenty-gram batches of whole-kernel corn were heated at 171°F for various periods. Ten grams were analyzed as whole-kernel corn. Each kernel of the remaining 10 g was separated into pericarp and endosperm-germ fractions by squeezing; the average separation was 40% pericarp and 60% endosperm-germ. The enzyme concentration of each fraction was expressed as percent activity of raw whole-kernel corn.

The results are shown in Fig. 2. The curves are only approximate because the

heating times were widely spaced. The heat-inactivation curves for the two fractions, pericarp and endosperm-germ, were similar to that for whole-kernel corn, showing an initial rapid enzyme inactivation followed by a slow rate.

The percent activity values obtained for pericarp and endosperm-germ showed totals in close agreement with those for whole kernels. The enzyme content of the raw pericarp fraction accounted for 54% of the total enzyme activity in raw whole-kernel corn. The resistant fraction in raw whole-kernel corn as determined by extrapolation was 5% of the total. The pericarp accounted for most of this resistant peroxidase fraction, 3.5% of the total activity or 78% of the resistance fraction, in whole-kernel corn. This gives a quantitative confirmation of observations made by Joslyn (1949).

Effect of temperature. Since the inactivation of peroxidase followed two first-order reactions, each straight-line segment or heat resistance should be considered separately in studying the effect of temperature.

Heat-inactivation curves similar to Fig. 1 were experimentally determined at ten-degree intervals over the range 150–200°F. A "D" value was calculated for each straight-line segment, and the results are plotted in Figs. 3 and 4 with D values on the logarithmic scale. The excellent agreement of D values as determined by the OPDA and ascorbic acid enzyme assays should be noted in both figures.

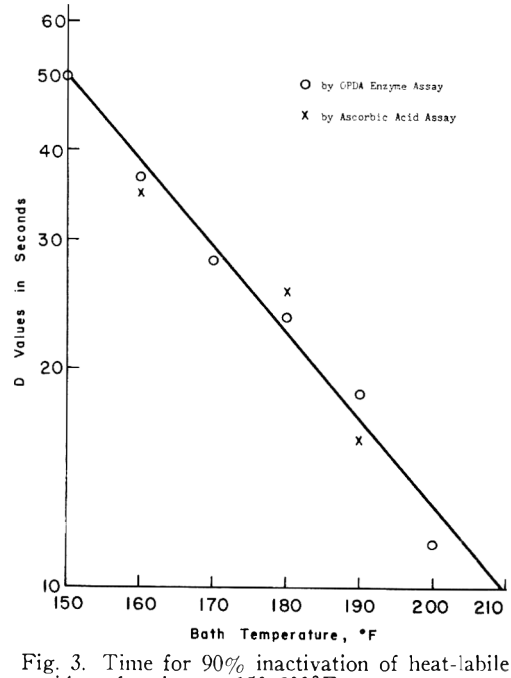


Fig. 3. Time for 90% inactivation of heat-labile peroxidase fraction at 150–200°F.

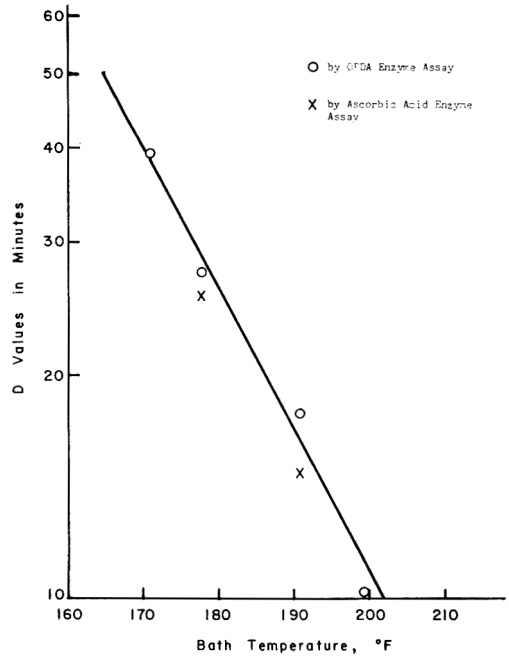


Fig. 4. Time for 90% inactivation of heat-resistant peroxidase fraction at 170–200°F.

Fig. 3 shows the variation of D value with inactivation temperature for the heat-labile fraction. This phantom curve approximated a straight line, having a Q_{10} of 1.6, a z value of 87°F, and a D value at 200°F of 13 sec. There were several sources of error in this curve. The extremely rapid inactivation necessitated the use of rate data before the kernels reached bath temperature. Another factor is the poor precision obtained with an experimental technique using such short heating times.

A straight line was also obtained for the heat-resistant fraction (Fig. 4). Experimental errors due to heat penetration lag were non-existent since heating time extended for relatively long periods at the experimental temperature. The Q_{10} value was 2.1, the z value was 54°F, and the D value at 200°F was 11 min.

The practical significance of these findings

is that, at the temperatures normally used for blanching, little peroxidase inactivation is gained by increasing blanch times past the point of rapid inactivation, i.e., where the inactivation curve changes to a shallow slope. This can be quantitatively shown by comparing the degree of inactivation accomplished by blanching times of 2 and 5 min. After 2 min at 200°F, the residual enzyme activity was only 3.3% of the original. Increasing the heating time to 5 min would leave residual activity of 1.7% of the total original activity. At the storage temperatures normally used for frozen whole-kernel corn it is questionable whether a difference in storage stability exists between 3.3 and 1.7% residual activity (Joslyn, 1949).

Inactivation at 210–290°F. It was shown earlier that peroxidase was inactivated in what appeared to be two first-order reactions. The inactivation of the heat-labile fraction was shown to be extremely rapid. In temperatures used for thermal processing of sweet corn the labile fraction is of little concern, and this portion of the study was therefore confined to the heat-resistant enzyme fraction.

Table 1 shows heat penetration into the

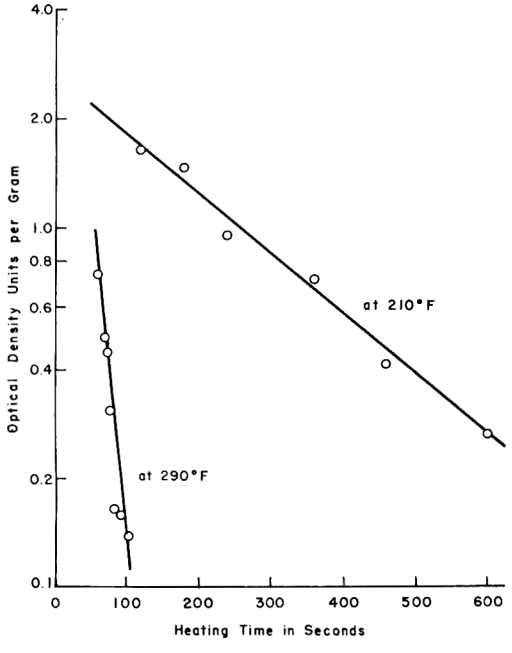


Fig. 5. Inactivation of peroxidase at 210 and 290°F in whole-kernel corn sealed in thermal death-time cans.

Table 1. Heat penetration into thermal death-time can.

Temp. of oil bath (°F)	Time to reach −5°F of bath temp. (sec)	Time to reach bath temp. (sec)
220	65 [a]	95 [a]
245	70 [a]	100 [a]
260	75	100
278	70	100
300	70	90

[a] The indentation was removed from the bottom of the thermal death-time cans.

TDT cans as determined by the recording potentiometer. It was shown earlier that the resistant fraction of peroxidase was located primarily in the pericarp. Since this was the case, it was felt that heat penetration lag into the center of the kernel could be neglected and that only the heat penetration lag into the TDT can need be considered. Whenever possible, rate calculations were based on data obtained after the internal temperature of the can reached bath temperature. At temperatures above 245°F, the rapid inactivation necessitated the use of a *range* of temperatures, including those during the latter part of the come-up time. The rate of inactivation was then expressed as the rate at the average internal temperature. This range never exceeded ±2.5°F.

The rates of inactivation were determined at 210, 225, 234, 245, 262, 275, and 290°F. Fig. 5 shows inactivation-time curves for 210 and 290°F. Only the OPDA method of enzyme analysis was used, since the ascorbic acid assay method gave essentially the same results in the earlier study.

Data for inactivation of the heat-resistant fraction in the temperature range studied indicated it to be a first-order reaction. The curves did not show the initial rapid inactivation, as with the earlier low-temperature study, because the activity of the heat-labile fraction was reduced to a negligible level during the come-up time.

Fig. 6 gives the D values calculated from plots of the data at each temperature. A straight line drawn through the points showed a Q_{10} of 1.9, a z value of 65°F, and a D value at 290°F of 0.75 min. Once this 90% inactivation line has been experimentally established a phantom curve for

any other degree of inactivation is easily obtained. This was done by drawing a line parallel to the first and displaced vertically on the time axis. Thus the 99% or 2D line was placed at two times the heating time for 1D at any given temperature. For illustration, phantom lines for 0.5D, 2D, and 3D were drawn in Fig. 6.

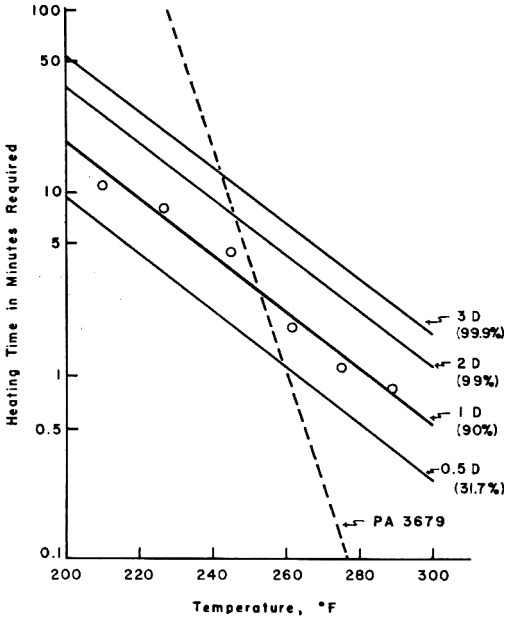


Fig. 6. Time for several D values of inactivation for the heat-resistant fraction of peroxidase as compared to thermal death times for spores of P. A. 3679.

The dotted line crossing the phantom curves in Fig. 6 is the TDT curve for the thermophilic spore-forming bacterium PA 3679, having an F_{250} value of 4.0 min and z value of 16.6°F (Townsend and Esty, 1938). This line, representative of those used to determine the minimum thermal process time for canned vegetables, has a markedly steeper slope than the enzyme inactivation curves. Fig. 6 shows that the heat-resistant peroxidase enzyme is over 99.9% inactivated if the product is sterilized at 240°F, but only 90% inactivated if barely sterilized at 260°F. Indeed, Guyer and Holmquist (1954) reported that it was often found necessary to increase high-temperature process times above calculated values to prevent off-flavors from developing in whole-kernel corn during extended storage.

The relation between the slopes of the phantom inactivation-time curve and of the heat inactivation-time curve is theoretically identical if a first-order reaction holds to the point where the assay fails to detect enzyme activity (Esselen and Pflug, 1954). The slope of the phantom curves was fairly consistent with that of the straight-line heat-inactivation curve for whole-kernel corn assayed immediately after heating as obtained by Vetter *et al.* (1959). They reported a z of 59°F, compared to 65°F found here. Of special interest is the fact that the curve obtained in this study showed no inflection point, as did the heat inactivation-time curve for samples stored 72 hr at 75°F to allow enzyme regeneration. A possible explanation for this difference may be that the storage temperature used in the present case was 34°F instead of 75°F. Another may be that a kinetic technique was used here instead of the "end-point" method.

REFERENCES

Ball, C. O., and F. C. W. Olson. 1956. "Sterilization in Food Technology." McGraw-Hill Book Company, Inc., New York.

Esselen, W. B., and I. J. Pflug. 1954. Thermal destruction of microorganisms and enzymes. *Glass Packer I.* **33**(1), 27; II. **33**(2), 28.

Farkas, D. F., S. A. Goldblith, and B. E. Proctor. 1956. Stopping storage off-flavors by curbing peroxidase. *Food Eng.* **28**(1), 52.

Guyer, R. B., and J. W. Holmquist. 1954. Enzyme regeneration in high temperature short time sterilized canned foods. *Food Technol.* **8**, 547.

Jermyn, M. A. 1952. Horseradish peroxidase. *Nature* London **169**, 488.

Joslyn, M. A. 1949. Enzyme Activity in Frozen Vegetable Tissue, *Advances in Enzymol.* **9**, 613.

Joslyn, M.A. 1956. Report on peroxidase in frozen vegetables. *J. Assoc. Offic. Agr. Chemists* **39**, 267.

Joslyn, M. A. 1957. *J. Assoc. Offic. Agr. Chemists* **40**, 338.

Stumbo, C. R. 1949. Thermobacteriology as applied to food processing. *Advances in Food Research*, **2**, 47.

Townsend, C. T., and J. R. Esty. 1938. Heat resistance studies on spores of putrefactive anaerobes in relation to determination of safe processes for canned foods. *Food Research* **3**, 323.

Vetter, J. L., A. I. Nelson, and M. P. Steinberg. 1958a. Heat inactivation of sweet corn peroxidase in the temperature range of 210° to 310°F. *Food Technol.* **12**, 244.

Vetter, J. L., M. P. Steinberg, and A. I. Nelson. 1958b. Quantitative determination of peroxidase in sweet corn. *J. Agr. Food Chem.* **6**, 39.

Vetter, J. L., A. I. Nelson, and M. P. Steinberg. 1959. Heat inactivation of peroxidase in HTST processed whole kernel corn. *Food Technol.* **13**, 410.

Yamamoto, H. Y. 1958. "Kinetics of heat inactivation of sweet corn peroxidase." M.S. thesis. University of Illinois Library, Urbana, Illinois.

Peroxidase Regeneration and Its Effect on Quality in Frozen Peas and Thawed Peas

B. R. W. PINSENT
Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford, England

(Manuscript received September 18, 1961)

SUMMARY

Peroxidase was regenerated in samples of peas that had been blanched just sufficiently to destroy the original peroxidase activity. The regeneration occurred after a few months in peas stored at −18° C, or within a few hours in thawed peas held at room temperature. The maximum activity regenerated was about 4% of the original activity. No regeneration occurred in peas given substantially more heat treatment than was needed for inactivation. Peroxidase regeneration during frozen storage, or after thawing, did not affect the quality of peas.

Peas are blanched before freezing to inactivate enzymes that would otherwise catalyse reactions that produce off-flavors. The normal commercial practice is to blanch for 60–90 seconds in water at 95–100°C. These conditions for adequate blanching are based on a great deal of published evidence (Joslyn 1949, Lee 1958) and practical experience. However, because of variations in temperature and pea-water ratio in blanchers, it is unwise to rely entirely on time of blanching, and a biochemical index of blanching is often used. The enzymes responsible for off-flavors are not yet identified, but the inactivation of catalase or peroxidase is generally taken to imply that peas have been blanched sufficiently, although these enzymes are probably not involved in off-flavor development. The merits of catalase and peroxidase as an index of blanching have been discussed by Joslyn (1949) and Lee (1958). Peroxidase has greater heat stability than catalase and is a safer index.

Some doubts as to the validity of using peroxidase as a blanching index have been raised by reports that peroxidase is regenerated in tissues after heat induction. These reports have been reviewed by Joslyn (1949), Schwimmer (1944) and Leeson (1957). Schwimmer (1944) found that peroxidase activity in blanched cabbage and turnip tissues increased after holding for a few hours at 6 or 25°C, but all his samples had some residual peroxidase activity immediately after blanching. Farkus *et al.* (1956) found a slight reactivation in peas at room temperature, even in tissues showing no activity immediately after blanching, and Rosoff *et al.* (1949) found that heat-inactivated cauliflower peroxidase reappeared during subsequent storage at 0 or −18°C. Reddi *et al.* (1950) found that apple peroxidase was not regenerated after it had been completely inactivated. Zoueil *et al.* (1959) found that regeneration of peroxidase could occur in green beans and turnips stored at 22 or 3°C after heating just sufficiently for inactivation, but no regeneration occurred after substantially more heating had been given.

Although there is abundant evidence of regeneration at temperatures of 0°C and above, the only reliable evidence of regeneration at commercial storage temperatures (about −18°C) is the work of Rosoff *et al.* (1949) on inactivated cauliflower peroxidases. Dietrich *et al.* (1955) measured the peroxidase activity of peas immediately after blanching, and after 6 and 12 months of storage at −18°C, and their results suggest that some regeneration occurred. However, they held the peas for 1½ hr between blanching and freezing and, as the authors suggest, much of the apparent regeneration may have occurred before the peas were frozen. Their view is supported by the

fact that considerable regeneration appears to have occurred in only 3 weeks at −24°C.

The available evidence of the effect of regenerated peroxidase on the quality of frozen vegetables is somewhat contradictory. Wagenknecht *et al.* (1958) found that although peroxidase was regenerated in some samples of peas after prolonged frozen storage, there was no off-flavor. When they added horseradish peroxidase to blanched pea slurries, off-flavors developed in storage at −18°C. Zoueil *et al.* (1959) found that added peroxidase produced off-flavors in green bean purees. In the experiments described by Dietrich *et al.* (1955), samples of peas in which regeneration occurred appear to have had some off-flavors but, after 12 months of storage at −18°C, there was no significant difference in flavor between the 180-sec samples (over-blanched, no peroxidase), 90-sec sample (adequately blanched, regenerated peroxidase), and 50-sec sample (inadequately blanched, residual peroxidase). However, since the samples were delayed between blanching and freezing for a time "far in excess of that which would be encountered under conditions of good commercial practice," some off-flavor may have developed before the peas were frozen.

The object of the present work was to try to obtain evidence of regeneration of peroxidase in blanched peas stored at −18°C, and to discover whether this affected the quality of the peas. It was also of interest to study the rate of peroxidase regeneration and its effect on flavor in thawed peas held at room temperature, since it is quite common practice, at least in Europe, for peas to be held at room temperature for up to 24 hr between purchase and cooking.

PEROXIDASE ASSAY METHODS

Qualitative assay. The method of Joslyn (1949) was used.

Quantitative assay. Two methods were used: 1) *Bedford and Joslyn method (1939).* This method proved to be unsuitable for measuring very low peroxidase activity.

2) *Colorimetric guaiacol method.* This method was a modification of Ponting and Joslyn's (1948)

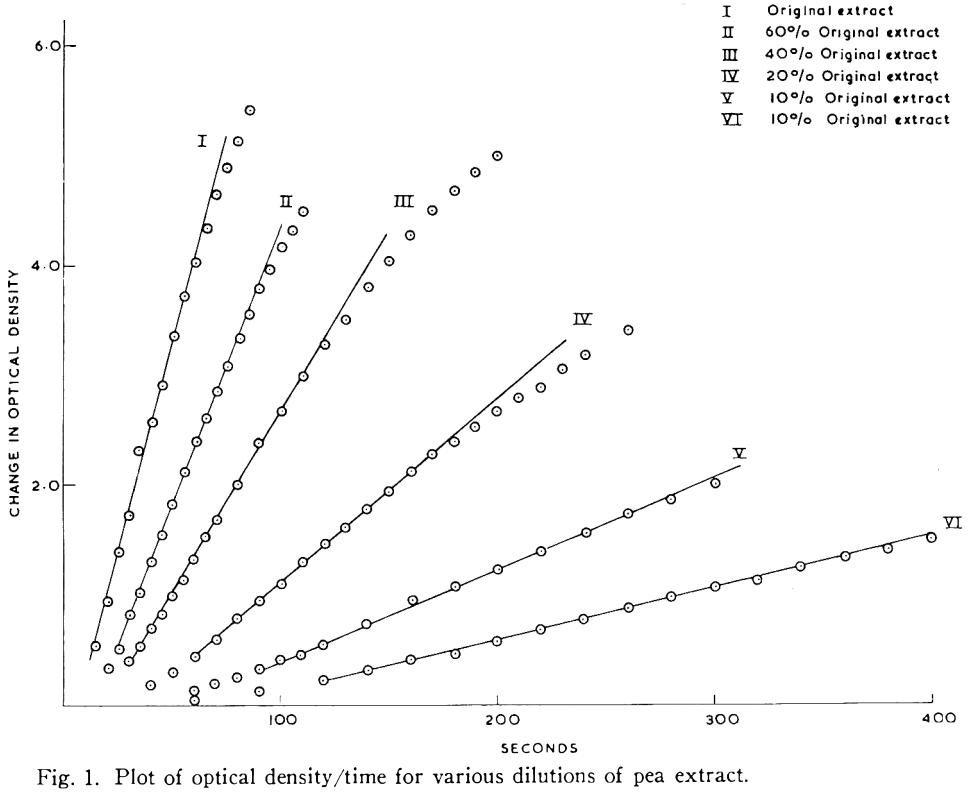


Fig. 1. Plot of optical density/time for various dilutions of pea extract.

method. It was compared with the ascorbic acid oxidation method (Joslyn 1957) and found to be equally reliable and more convenient for this work. The reagents used were: 0.2*M* acetate buffer (pH 5.58), 0.5% aqueous guaiacol solution, 0.05*N* hydrogen peroxide, 2% sodium chloride. All solutions prepared with glass distilled water. All tubes and pipettes must be cleaned very carefully.

Extraction. Fifty g of peas were blended for 3 min in an Atomix with 200 ml ice-cold sodium chloride solution plus a pinch of chalk. The extract was filtered through raw cotton and muslin on a filter pump. The filtrate was assayed immediately or stored at −18°C. The filtrate was diluted as required.

Assay. All reagents were kept at 20°C in a water bath. Five ml water and 2 ml acetate buffer were pipetted into an Eel colorimeter tube. One ml guaiacol solution, 1 ml pea extract, and 1 ml water were added. The tube was placed in the Eel colorimeter and the meter was set at 0. Another tube was made up with the same mixture of reactants but omitting the last 1 ml water. One ml hydrogen peroxide solution was added and mixed, a stopwatch was started and the tube was placed in the colorimeter. Readings were taken at convenient intervals. Optical density was plotted against time, and the slope of the line was measured after the initial lag period.

Fig. 1 shows plots for runs on several dilutions of an extract from peas. Except at the highest concentrations there was an initial lag period of 40–100 sec, but after this a linear plot was obtained for 60–300 sec. Fig. 2 shows the slopes plotted against the original activity. The rate of reaction was proportional to the enzyme concentration over a wide range of activity. The slopes could be determined accurately within the range 0.01–0.05 but above 0.05 they were too steep and it was advisable to dilute the more active extracts.

The method was calibrated against the Bedford and Joslyn method so that the results could be expressed in Willstatter purpurogallin units (PE units) per g of peas. One unit of slope (change in optical density/sec) was equivalent to 0.92×10^{-2} PE units. The method was convenient and reliable even at very low enzyme activities. It was not affected by ascorbic acid, or inhibited by hydrogen peroxide.

Taste-panel assessment. The quality of the samples was assessed by a taste-panel at intervals of a few months, up to 27 months. No single control could be used for all these tests, so the samples were scored without reference to a control, by a panel of trained tasters. The samples were scored on a numerical scale of 0–10, from inedible to superlatively good. It is unusual for peas to be marked above 7; 5–6 indicates an average quality, and scores below 4.5 indicate a definite lack of quality. A difference of one unit is necessary to denote a significant difference in quality.

Table 1. Peroxidase activity and flavor of pea stored at −18°C.

Blanching time (sec)	Storage time (months)					
	0	1½	2	4½	7½	17
Qualitative peroxidase assays						
0	+	+	+	+	+	+
60	−	−	+	+	+	+
90	−	−	−	−	−	−
120	−	−	−	−	−	−
180	−	−	−	−	−	−
	4	7½	17	21		
Quantitative peroxidase activity (PE units × 10^4/g peas) (values below 5 indicate no peroxidase activity)						
0	250	330	170	240		
60	11	8	9			
90	3	1	1	3		
120	2	2	2			
180			1			
	3¼	6	13½	17	22	27
Taste-panel flavor scores						
0	0	−	−	−	−	−
60	5.5	4.9	4.7	5.0	−	−
90	5.7	5.5	4.9	−	4.8	4.0
120	5.3	5.3	4.9	−	−	−
180	5.2	4.5	4.4	−	−	−

EXPERIMENTAL AND RESULTS

Peroxidase regeneration during storage of adequately blanched peas. Samples from a batch of Dark Skinned Perfection peas were blanched in a rotary blancher for 60, 90, 120, or 180 sec, frozen immediately after blanching, and stored at −18°C. The blancher temperature was 98–100°C, and the pea-water ratio was 1:20. A sample of unblanched peas was also frozen and stored.

Peroxidase was assayed at intervals of a few months. Samples were taken from the cold store, thawed, and immediately extracted and assayed. The taste-panel assessed flavor every few months. Results are in Table 1.

Peroxidase was completely inactivated in all samples immediately after blanching, and no peroxidase was regenerated in any samples blanched for 90 sec or longer. Some peroxidase activity was detectable after 2 months in the 60-sec sample, and quantitative assays after 4 months showed that this sample had about 4% of the peroxidase

activity of unblanched peas. The activity did not increase on further storage.

The taste-panel scores give a general picture of a gradual loss in quality of all the blanched samples during frozen storage. The decline in flavor score was due to a loss of natural flavor rather than to any off-flavor development. The 60-sec sample, in which there was evidence of peroxidase regeneration, received about the same flavor scores as the other samples. The unblanched sample was inedible at 3 months of storage.

This series of experiments was repeated on another batch of peas, blanched for 60, 90, and 120 sec. No peroxidase was detected in any sample by qualitative tests immediately after blanching, but when the peas were left at room temperature after blanching, peroxidase was regenerated in the 60-sec and 90-sec samples. Samples were frozen immediately after blanching and stored at −18°C. Peroxidase was assayed after 5 and 10 months of storage, and the taste-panel assessed flavor, color, and texture at intervals up to 17 months of storage. Results are in Table 2.

The 60-sec sample showed slight peroxidase activity after 5 months, and there were signs of very slight activity in the 90-sec sample after 10 months of storage. The taste-panel scores show a gradual decline in quality during storage, with no significant differences among the three samples.

Table 2. Peroxidase activity and quality of stored frozen peas stored at −18°C.

	Blanching time (sec)	Storage time (months)				
		2	5	10	15	17
Peroxidase activity PE units × 10^4 per g peas [a]						
	60	....	5	8	....	....
	90	....	4	6	....	....
	120	....	3	3	....	....
Flavor	60	5.9	5.7	5.5	5.5	4.8
	90	5.9	4.5	5.4	5.0	4.8
	120	6.1	5.3	5.2	4.7	4.7
Texture	60	5.7	5.3	5.3	4.5	4.9
	90	5.9	5.5	5.3	4.5	5.2
	120	5.9	5.5	4.9	4.5	4.9
Color	60	7.0	6.1	5.8	6.0	5.4
	90	6.6	6.2	5.5	6.1	5.0
	120	6.3	6.2	5.2	6.0	5.3

[a] Values below 5 indicate no peroxidase activity.

Peroxidase regeneration in thawed peas at room temperature. Regeneration of peroxidase at room temperature was followed quantitatively with the modified Ponting and Joslyn method. A carton of

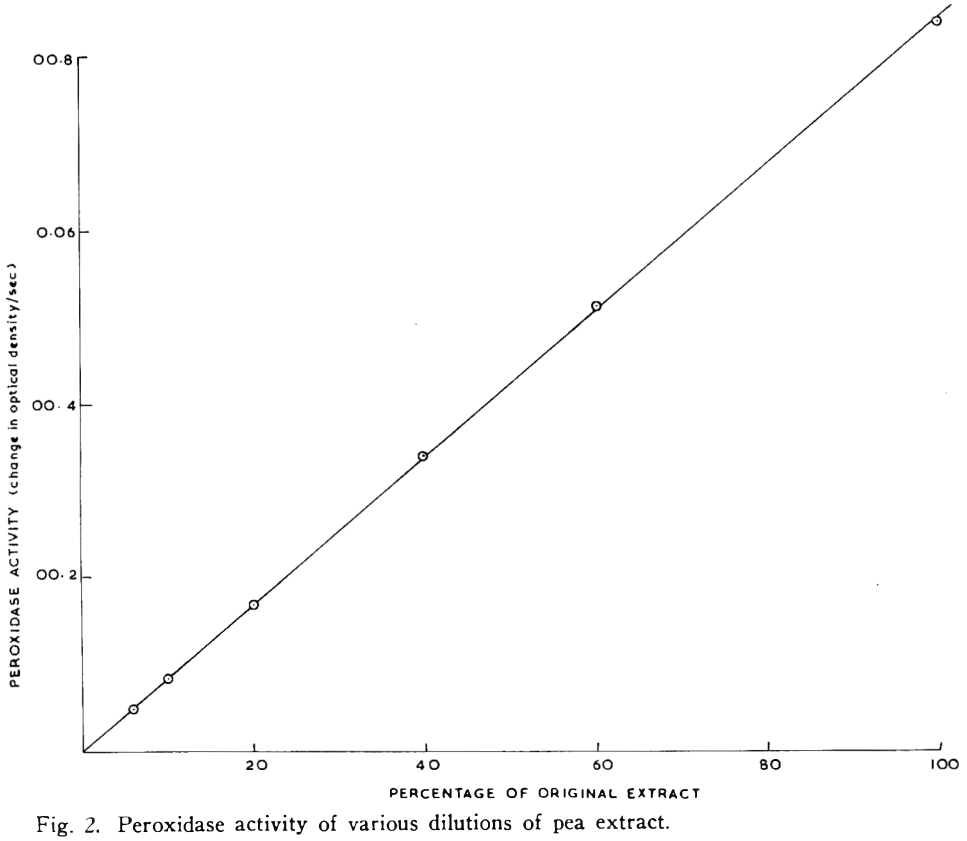


Fig. 2. Peroxidase activity of various dilutions of pea extract.

frozen peas (90-sec blanch) was taken from cold storage. The contents were placed in a flask which was stoppered with a raw cotton plug, and samples from the flask were assayed at intervals of a few hours. The time at which off-odors were first detected in the peas was noted. The results are in Fig. 3.

The peroxidase activity increased rapidly for the first few hours and reached a maximum at 30 hr, at which time the off-odor first became noticeable. The activity then decreased for a few hours and at 45 hr it reached a constant value of about 75% of maximum activity. Extracts of peas, obtained immediately after thawing, also increased in peroxidase activity on standing at room temperature, but less than the peas themselves.

The possible effect of this regeneration on quality was tested. A taste-panel compared a sample cooked 1 hr after removal from cold storage, with a sample cooked 18 hr after removal from cold storage. The peroxidase activity of these samples was also measured. The results are in Table 3. Although the 18-hr sample contained three times as much peroxidase as the 1-hr sample, the taste panel could not detect any difference in quality.

It was thought that regeneration of peroxidase at room temperature might be due to bacterial contamination. To investigate this possibility, blanched samples were soaked 20 min in solutions of mercuric chloride (1%) or streptomycin/penicillin (100 μg of each/ml) and left overnight at room temperature. This treatment should have destroyed any bacteria present, but these samples developed peroxidase activity as rapidly as untreated samples. To determine whether peroxidase was regenerated throughout the peas or only on the surface, samples of frozen peas were thawed and held overnight at room temperature. The peas were washed in $2N$ sodium chloride solution, and peroxidase was assayed in the peas and the washings. The washings contained only 5% of the total activity, and therefore the peroxidase was not being formed only on the surface of the peas.

Table 3. Peroxidase activity and quality of thawed peas.

Sample	Holding time (hr)	Peroxidase activity (PE units $\times 10^4$/g)	Taste-panel score		
			Flavor	Texture	Color
1	1	6	5.6	5.7	6.1
2	18	18	5.8	6.1	6.1

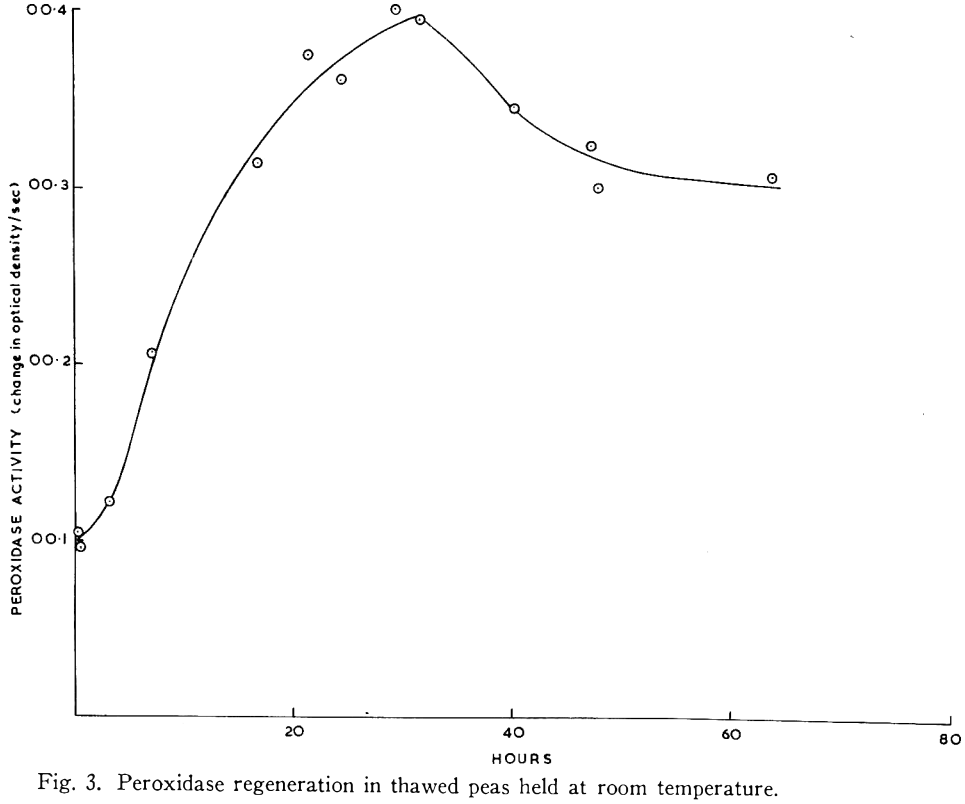


Fig. 3. Peroxidase regeneration in thawed peas held at room temperature.

These results make it improbable that peroxidase is formed by bacterial activity.

Storage properties of inadequately blanched peas. Samples from a batch of Kelvedon Wonder peas were pan-blanched for 45, 60, 75, and 90 sec, frozen immediately after blanching, and stored at −18°C. The blancher temperature was 98–100°C and the pea-water ratio was 1:20. Peroxidase was assayed qualitatively immediately after blanching, and the taste-panel assessed flavor at 4, 6, and 10½ months of storage. In an additional test at 10½ months the tasters were asked to ignore the natural flavors, and to state whether the samples had any off-flavor. Results are summarized in Table 4.

Table 4. Quality of peas stored at −18°C.

Blanching time (sec)	Peroxidase after blanching	Flavor scores at: 4 months	6 months	10½ months	Off-flavor after 10½ months
45	+	5.1	4.5	3.6	+
60	−	5.6	5.5	5.1	−
75	−	5.7	5.2	4.9	−
90	−	5.8	5.5	5.3	−

The 45-sec sample showed peroxidase activity immediately after blanching. It deteriorated far more rapidly than the other samples, and had a marked off-flavor after 10½ months of storage. Although peas keep satisfactorily despite peroxidase regeneration during storage, peas which have residual peroxidase immediately after blanching may develop off-flavors.

DISCUSSION

These results show that some peroxidase is regenerated in blanched peas during storage at −18°C. Earlier workers had shown that regeneration occurred in peas above 0°C, but there was no previous evidence of regeneration at −18°C. In this respect pea peroxidase behaves similarly to cauliflower peroxidase. Peroxidase regeneration occurred only in peas that had a heat treatment just sufficient to inactivate the enzyme. No regeneration occurred in peas given substantially more heat treatment than was needed for inactivation. The regenerated activity was never greater than 4% of the original activity. Peroxidase was also regenerated in thawed peas after holding at room temperature for a few hours, and the maximum regenerated activity was also about 4% of the original activity.

The extent of peroxidase regeneration is in reasonable agreement with the 7% found by Dietrich *et al.* (1955) in samples (90-sec) blanched just sufficiently for complete inactivation. The regeneration in their samples almost certainly occurred between blanching and freezing and thus it appears that the same amount of peroxidase is regenerated whether the regeneration occurs before, during, or after storage at −18°C.

Regenerated peroxidase did not affect the quality of the peas. All adequately blanched samples showed a decline in quality on storage, but this was due to loss of natural flavor, and not to the development of off-flavor. Samples lost quality at the same rate whether or not they contained regenerated peroxidase. Peas that had not been blanched sufficiently, and contained residual peroxidase immediately after blanching, developed off-flavors and deteriorated more rapidly than adequately blanched peas.

In our experiments, 60-sec blanching was just sufficient to inactivate all the peroxidase. Dietrich *et al.* (1955) found residual peroxidase even after 70-sec blanching, even though they used the Masure and Campbell (1944) qualitative assay, which is less sensitive than Joslyn's (1949) assay. This difference is due most probably to the lower blanching temperature and higher pea-water ratio used by Dietrich *et al.* (1955). This illustrates the danger of assuming a "safe" blanching time, and underlines the necessity for a biochemical index of adequate blanching.

REFERENCES

Bedford, C. L., and M. A. Joslyn. 1939. Enzyme activity in frozen vegetables—string beans. *Ind. Eng. Chem.* **31**, 751.

Dietrich, W. C., F. E. Lindquist, G. S. Bohart, H. J. Morris, and M. O. Nutting. 1955. Effect of degree of enzyme inactivation and storage temperature on quality retention in frozen peas. *Food Research* **20**, 480.

Farkus, D. F., S. A. Goldblith, and B. E. Proctor. 1956. Stopping storage off-flavors by curbing peroxidase. *Food Eng.* **28**(1) 52.

Joslyn, M. A. 1949. Enzyme activity in frozen vegetable tissue. *Advances in Enzymol.* **9**, 613.

Joslyn, M. A. 1957. Report on peroxidase in frozen vegetables. *J. Assoc. Offic. Agr. Chemists.* **40**, 338.

Lee, F. A. 1958. The blanching process. "Advances in Food Research," **8**, 63.

Leeson, J. A. 1957. "The Inactivation of Enzymes by Heat." The Fruit and Vegetable Canning and Quick-Freezing Research Association, Chipping Campden, Gloucestershire, U.K. Sci. Bull No. 2.

Masure, M. P., and H. Campbell. 1944. Rapid estimation of peroxidase in vegetable extracts—an index of blanching adequacy for frozen vegetables. *Fruit Prods. J.* **23**, 369.

Ponting, J. B., and M. A. Joslyn. 1948. Ascorbic acid oxidation and browning in apple tissue extracts. *Arch. Biochem.* **19**, 47.

Reddi, R. R., W. B. Esselen, and G. R. Fellers. 1950. Peroxidase activity of apple tissue. *Food Technol.* **4**, 63.

Rosoff, H. D., and W. V. Cruess. 1949. The oxidase of cauliflower. *Food Research.* **14**, 283.

Schwimmer, S. 1944. Regeneration of heat inactivated peroxidase. *J. Biol. Chem.* **154**, 487.

Wagenknecht, A. C., and F. A. Lee. 1958. Enzyme action and off-flavor in frozen peas. *Food Research*, **23**, 25.

Zoueil, M. E., and W. B. Esselen. 1959. Thermal destruction rates and regeneration of peroxidase in green beans and turnips. *Food Research* **24**, 119.

Ascorbic Acid Oxidation and Lipid Peroxidation in Milk

GRAFTON J. SMITH[a] AND W. L. DUNKLEY
Department of Food Science and Technology, University of California, Davis, California

(Manuscript received October 14, 1961)

SUMMARY

Ascorbic acid oxidation was studied in relation to the natural copper level and to lipid peroxidation in milks produced by cows receiving alfalfa hay or pasture. Correlations were highly significant ($r = 0.79^{***}$) between ascorbic acid oxidation rate and natural copper for both alfalfa and pasture milks. The correlation between lipid peroxidation (by the TBA test) and ascorbic acid oxidation rate was very highly significant ($r = 0.87^{***}$) in alfalfa milks but nonsignificant in pasture milks.

Some postulated mechanisms for the action of copper and ascorbic acid in producing oxidized flavor were studied. The results do not support the theories of the role of the ascorbic acid radical, or of hydrogen peroxide, in the initiation of oxidized flavor.

The kinetics of ascorbic acid oxidation by light, hydrogen peroxide, and ascorbic acid oxidase were examined. Zero-order kinetics was found for light, and first-order for the other agents.

Milk and some other dairy foods are subject to oxidative rancidity, commonly called oxidized flavor. Comprehensive reviews on this subject have been published by Strobel *et al.* (1953) and Riel and Sommer (1954). It is generally accepted that the flavor results from oxidation of unsaturated fatty acids in the phospholipids (Swanson and Sommer, 1940), and that copper and ascorbic acid are important prooxidants (Krukovsky and Guthrie, 1945; Smith and Dunkley, 1961). Several investigators have reported a positive correlation between the rate of ascorbic acid oxidation and oxidized flavor development (Riel and Sommer, 1954). King (1958), however, believed that oxidized flavor did not necessarily occur at intermediate or higher rates of ascorbic acid oxidation.

The behavior of the ascorbic acid in relation to oxidized flavor is anomalous. At concentrations above those in normal milk, ascorbic acid acts as an antioxidant (Riel and Sommer, 1954), but at lower concentrations it is a prooxidant. Oxidized flavor can be prevented by rapid destruction of ascorbic acid (Krukovsky and Guthrie, 1945).

A mechanism for oxidized flavor development involving both ascorbic acid and hydrogen peroxide was proposed by Brown and Olson (1942). They postulated that ascorbic acid reduces cupric copper to cuprous, the cuprous ion is oxidized to cupric by molecular oxygen with the production of hydrogen peroxide, and the hydrogen peroxide oxidizes the lipids in the fat globule membrane.

Krukovsky and Guthrie (1946) suggested that oxidation of ascorbic acid is coupled to the lipid oxidation reactions when a certain equilibrium has been established between ascorbic and dehydroascorbic acids. In their experiments they prevented the development of oxidized flavor by adding hydrogen peroxide to destroy ascorbic acid. The action of hydrogen peroxide depended on the concentration added, and under carefully controlled conditions it induced oxidized flavor in some samples. An enzyme, later shown to be peroxidase (Krukovsky, 1949), promoted the destruction of ascorbic acid on addition of hydrogen peroxide.

Other explanations have been given for the prooxidant role of ascorbic acid in oxidized flavor. Greenbank (1940) considered that E_h poising is important in determining

[a] Present address: Hawkesbury Agricultural College, Richmond, N.S.W., Australia.

whether oxidized flavor develops, and that the effect of ascorbic acid is dependent on its poising action. The ascorbic acid radical has been postulated as a peroxidation initiator—in milk by King (1958), and in other materials by Kelly and Watts (1957). Smith and Dunkley (1961) considered that the prooxidant properties of ascorbic acid are attributable to two properties—the ability to reduce cupric copper to the cuprous form, and a specific association between ascorbic acid and copper that in some unexplained manner increases prooxidant activity.

An investigation was made to clarify understanding of the relation of ascorbic acid oxidation to lipid oxidation in milk.

METHODS AND MATERIALS

Milk. Unless otherwise specified, the milks used were individual milks from cows maintained on a ration of alfalfa hay and concentrate mixture (barley and milo grain, wheat-mixed feeds, and molasses beet pulp). In some instances, samples were obtained from cows receiving irrigated legume-grass pasture. The milk was protected from light, and precautions to avoid metal contamination were taken according to the method of Smith and Dunkley (1961).

Heat treatment of milk. All samples, except those used for Figs. 5, 6, and 7, were heated 20 min. at 60°C in pint polyethylene bottles. The heat treatment was used to inactivate lipase, to retard bacterial spoilage, and in some cases to convert dehydroascorbic acid to 2,3-diketogulcnic acid. The conversion of dehydroascorbic acid to 2,3-diketogulonic acid was necessary to prevent re-formation of reduced ascorbic acid.

Oxidation of ascorbic acid. Added crude ascorbic acid oxidase, light, and hydrogen peroxide were studied as promoters of ascorbic acid oxidation in milk. Crude ascorbic acid oxidase was prepared from cucumbers by the method of Stewart and Sharp (1945). For the light treatment, 500 ml of milk in a square quart glass milk bottle were exposed with agitation, at a distance of ½ in. from two 40-watt cool white fluorescent lamps. Hydrogen peroxide for the activation energy experiments was added at the rate of 0.3 ml (30% Merck reagent) per liter of milk. In the other experiments, 0.02–0.2 ml (30% Mallinckrodt AR) was used per liter.

Ascorbic acid. D- and L-ascorbic acid (Eastman) were used.

ATA 3-amino-1,2,4-triazole (twice recrystallized from absolute ethanol). A $10^{-2}M$ solution was made in the milk. This level inhibits peroxidase, but not catalase, over the first two hours of reaction (Castelfranco, 1960).

Catalase. Lyophilized beef-liver catalase (N. B. Co.) was added at the rate of 5 mg per 200 ml milk.

Organoleptic scoring. Flavor evaluations were made by at least two competent judges. The results of organoleptic examinations are expressed as average flavor scores indicating intensity of oxidized flavor: 0, none; 1, questionable to very slight; 2, slight but consistently detectable; 3, distinct or strong; 4, very strong.

Thiobarbituric acid (TBA) test. The Dunkley and Jennings method (1951) was used. To overcome the problem of cloudiness in the cuvettes, the extracted chromophore was filtered (Whatman No. 12 fluted) before absorbancy was measured in the Beckman spectrophotometer. Results are expressed as the increase in absorbancy over that for the fresh milk.

Ascorbic acid. The method of Sharp (1938) was used except that the dye solution was prepared by dissolving 0.135 g of sodium 2,6-dichlorophenolindophenol (Eastman) in hot water and diluting to 1 liter.

Statistical analyses were by conventional methods (Snedecor, 1956).

RESULTS AND DISCUSSION

Copper-catalyzed ascorbic acid oxidation. Fig. 1 gives representative results illustrating the rate of ascorbic acid oxidation in alfalfa-fed milks. In samples with the slowest initial rates, there were usually two

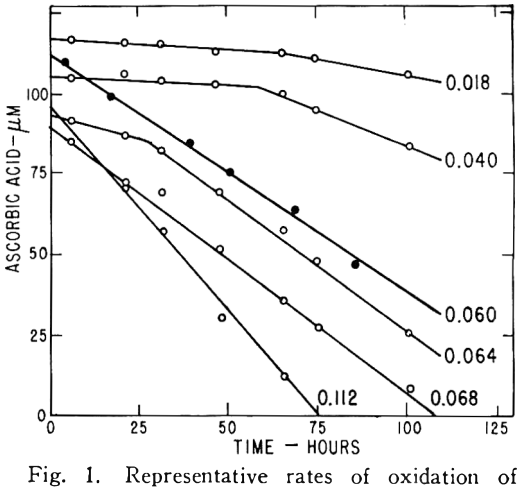


Fig. 1. Representative rates of oxidation of ascorbic acid at 3–4°C in milks from individual cows. Figures on curves indicate natural copper content (μg/g). Solid circles indicate early lactation (6 weeks or less).

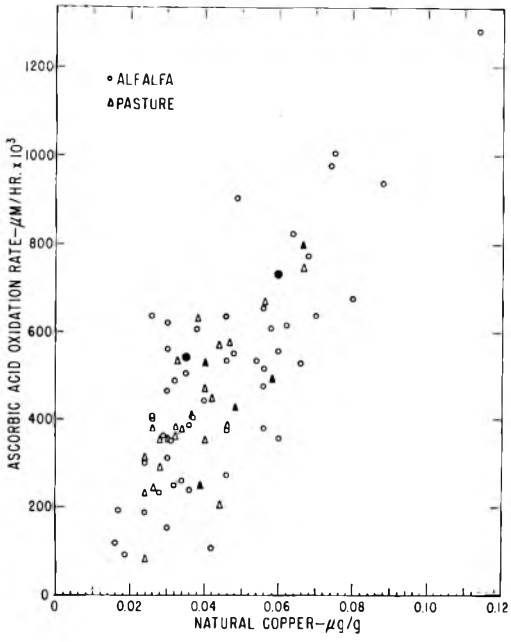


Fig. 2. Relation between rate of ascorbic acid oxidation (3–4°C) and natural copper content of milks from individual cows on either an alfalfa ration (circles) or on pasture (triangles). Solid points represent early-lactation milks (6 weeks or less).

zero-order rates—a slow initial rate and a faster second rate, with a sharp break between. This phenomenon was also noted in pasture-fed milks. The time required for the change in oxidation rate was significantly correlated ($r = -0.44^*$) with the natural copper content of milk. The change in rate may be due to disappearance of an antioxidant that is preferentially oxidized before the ascorbic acid. Holmes (1952) observed a small decrease in ascorbic acid loss during the storage of milk with added tocopherol. The difference in rate was much smaller, however, than illustrated for the individual milks in Fig. 1.

Fig. 2 illustrates the relation between the rate of ascorbic acid oxidation and natural copper content of milk from cows on alfalfa ration and cows on pasture. For the samples in which the ascorbic acid oxidation curve showed a break, the faster (second) rate was plotted. There was a highly significant correlation ($r = 0.79^{***}$) between the faster ascorbic acid oxidation rate and the natural copper content of the milks, but low correlation ($r = 0.18$) between 16 slow (first) rates and natural copper. No differences were related to the rations. If milk produced by pasture feeding contained more antioxidants than milk produced on the alfalfa hay ration, the difference did not influence the rate of ascorbic acid oxidation.

The kinetics illustrated in Figs. 1 and 2 are not consistent with those of related systems described by others. Ascorbic acid oxidation in milk showed zero-order dependency on ascorbic acid, and the rate was directly proportional to the copper content. For ascorbic acid oxidation in buttermilk, Allan (1950) showed first-order dependency on ascorbic acid and direct proportionality to copper content. Timberlake (1960) found that ascorbic acid oxidation in a pH 2.9 model system was first-order, and that the rate was proportional to the square root of the copper concentration. Since ascorbic acid has been shown to exist in bound form

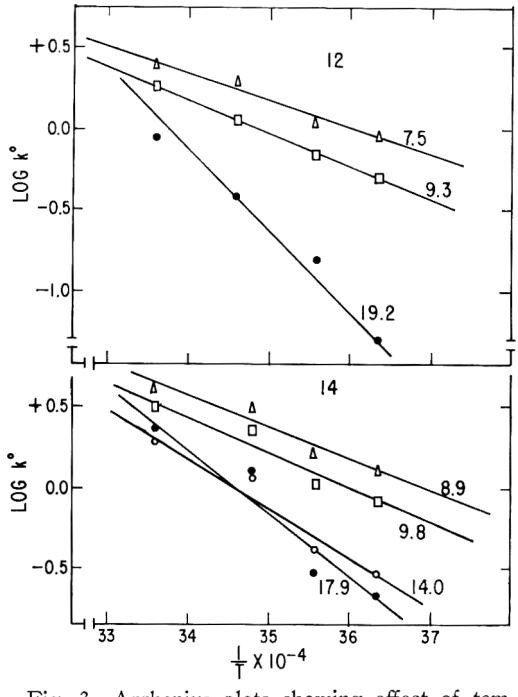


Fig. 3. Arrhenius plots showing effect of temperature on rate of oxidation of ascorbic acid catalyzed by natural and added copper for two cows (nos. 12 and 14). ○—○ normal milk; ●—● buttermilk added (to increase natural copper); □—□ 0.05 μg/g copper added to milk; △—△ 0.10 μg/g copper added to milk. Figures on curves indicate activation energies (kcal/mole).

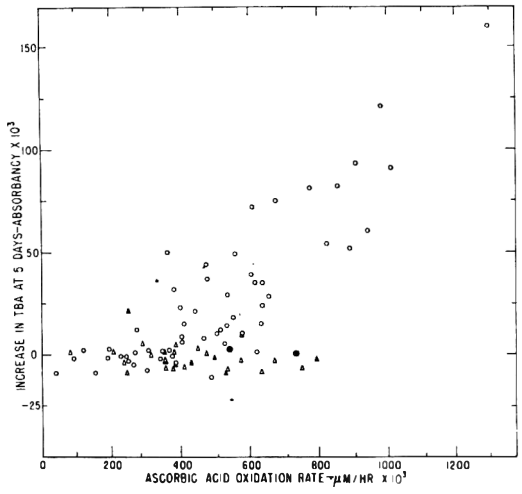


Fig. 4. Relation between TBA absorbancy increase and rate of ascorbic acid oxidation in milks from individual cows on an alfalfa ration (circles) or on pasture (triangles). Solid points represent early-lactation milks (6 weeks or less).

(Greenberg, 1957), the difference in reaction order between milk and either buttermilk or the Timberlake model may be due to exclusion of ascorbic acid from the rate-determining step by steric hindrance.

Natural and added copper were compared with respect to the influence of temperature on catalysis of ascorbic acid oxidation. Chloroform was added to the milk to inhibit bacterial spoilage at the higher temperatures. Fig. 3 shows the results for two milks. The activation energy was lower in samples containing added copper than in samples containing only natural copper. Natural copper was increased by adding buttermilk prepared from the same milk; this increased the activation energy.

The difference in activation energy for natural and added copper indicates a difference in chemical binding. Allan (1950), working with buttermilk and butter serum, also reported a lower activation energy for added copper than for natural copper. He concluded that the natural and added copper were associated with the same proteins but were attached in a different manner. King *et al.* (1959), in fractionation studies of milk, concluded that natural copper was preferentially associated with the fat-globule membrane, whereas added copper was largely distributed in proportion to the composition of the skimmilk proteins.

Ascorbic acid oxidation in relation to lipid peroxidation. Fig. 4 illustrates the relation between TBA absorbancy increase and ascorbic acid oxidation rate in 58 alfalfa-fed and 28 pasture-fed milks. For the alfalfa-fed milks, the correlation was very highly significant ($r = 0.87^{***}$) when the two early-lactation results were excluded. For the pasture-fed milks there was no significant correlation. As indicated by the low TBA-absorbancy increases, the pasture milks were more resistant to oxidized flavor than the alfalfa milks. Despite the high correlation between rate of ascorbic acid oxidation and TBA absorbancy increase in alfalfa milks, it is not the ascorbic acid oxidation rate that determines the rate of lipid oxidation.

Kinetics of ascorbic acid oxidation by light, hydrogen peroxide, and ascorbic acid oxidase. Fig. 5 shows representative ascorbic acid oxidation plots. Zero-order kinetics was obtained for light catalysis, but first-order kinetics applied for hydrogen peroxide and ascorbic acid oxidase. For the reaction catalyzed by ascorbic acid oxidase, the increase in rate after about 60 min may have resulted from activation by proteins, calcium, or both (Frieden and Maggiolo, 1957), even though the enzyme may be undergoing reaction inactivation over a prolonged period (Dawson and Magee, 1955). The catalytic activity of the enzyme was much greater

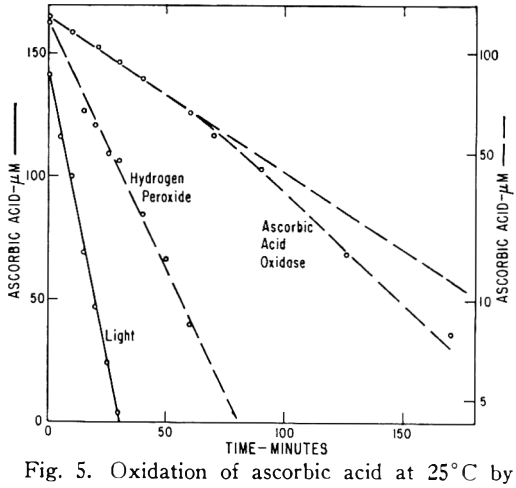


Fig. 5. Oxidation of ascorbic acid at 25°C by light, hydrogen peroxide, and ascorbic acid oxidase.

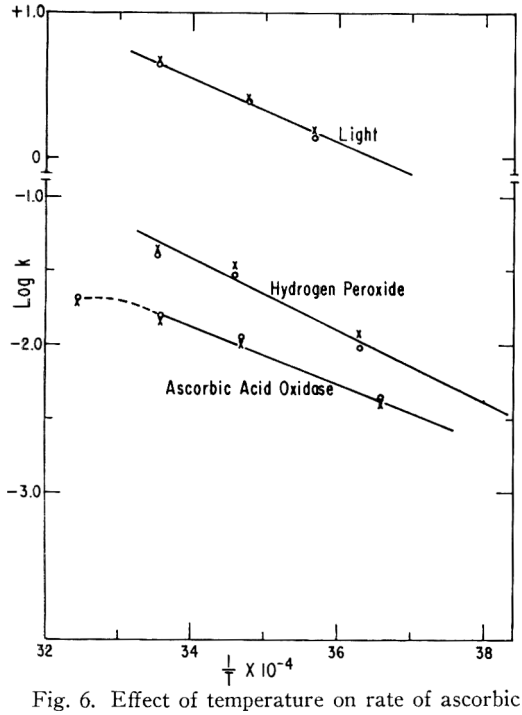


Fig. 6. Effect of temperature on rate of ascorbic acid oxidation catalyzed by light, hydrogen peroxide, and ascorbic acid oxidase. Data for two milks.

than would have been obtained by adding to milk an amount of copper corresponding to that added as part of the enzyme preparation (less than 0.001 μg per g).

Fig. 6 shows the effect of temperature on the oxidation of ascorbic acid in two milks. The respective activation energies for light, hydrogen peroxide, and ascorbic acid oxidase catalysis were 9.8, 11.2, and 9.0 kcal per mole. At a temperature above the optimum (25°C) for ascorbic acid oxidase, the reaction rate decreased with time.

Relation of hydrogen peroxide to ascorbic acid oxidation and lipid peroxidation. Variations have been noted (Krukovsky and Guthrie, 1946) in the action of hydrogen peroxide in the rapid destruction of ascorbic acid. When the concentration of hydrogen peroxide was increased from $1.76 \times 10^{-4}M$ to $1.76 \times {}^{-3}M$ (0.02 to 0.2 ml/L), ascorbic acid oxidation was inhibited (Fig. 7). A logical explanation is an inactivation of an enzyme involved in hydrogen peroxide breakdown of ascorbic acid. The specific peroxidase inhibitor ATA substantially prevented ascorbic acid oxidation by hydrogen peroxide, indicating that peroxidase catalyzes the oxidation. This confirms the results of Krukovsky's (1949) experiments in which hydrogen peroxide oxidation of ascorbic acid was prevented by heat treatment, and ascorbic acid oxidation was rapid on addition of horse-radish peroxidase. Lactoperoxidase is inactivated by the heat treatment.

Olson and Brown (1942) postulated that the reactant that initiates the attack on the lipid in the development of oxidized flavor in milk is hydrogen peroxide formed by copper-catalyzed oxidation of ascorbic acid. Table 1 gives representative results of experiments designed to test this hypothesis.

The addition of catalase did not slow ascorbic acid oxidation or lipid peroxidation. If hydrogen peroxide formed during oxidation of ascorbic acid is necessary to promote lipid oxidation, the catalase should have prevented lipid peroxidation by destroying the hydrogen peroxide.

Very low levels of added hydrogen peroxide ($1.76 \times 10^{-4}M$) had variable effects on

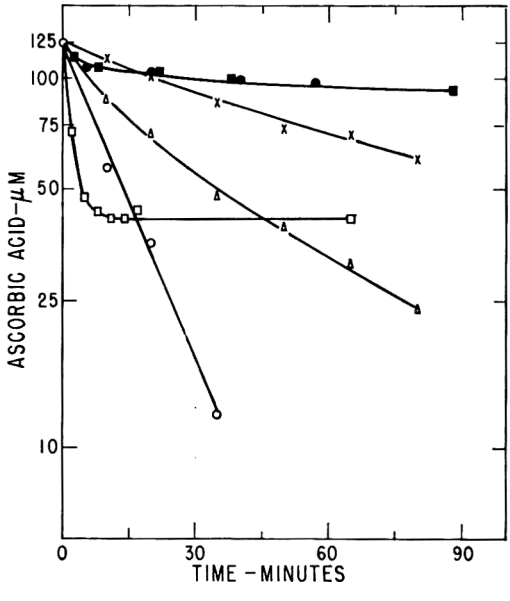


Fig. 7. Effect of hydrogen peroxide concentration and peroxidase inhibitor on the oxidation of ascorbic acid at 0°C.. □—□ $1.76 \times 10^{-4}M$ hydrogen peroxide; ○—○ $3.53 \times 10^{-4}M$ hydrogen peroxide; △—△ $7.06 \times 10^{-4}M$ hydrogen peroxide; X—X $1.76 \times 10^{-3}M$ hydrogen peroxide; ■—■, ●—● as above, but with $10^{-2}M$ ATA.

Table 1. The effect of hydrogen peroxide on ascorbic acid and lipid oxidation in milk at 3–4°C.

Sample	Ascorbic acid oxidation rate during storage (k^0, μM/hr × 10^2)	TBA increase at 5 days (absorbancy × 10^3)	Flavor score at 5 days
17	98	120	4
+ catalase	93	113	4
+ H_2O_2[a]	0[b]	3	0
AA destroyed[c]	0	−2	0
AA destroyed[c] + H_2O_2	0	40	0
18	53	5	0
+ catalase	56	16	0
+ H_2O_2	22	26	2
AA destroyed[c]	0	3	0
AA destroyed[c] + H_2O_2	0	8	0

[a] 1.76×10^{-4} M.
[b] The ascorbic acid was all oxidized within 10 hr.
[c] The ascorbic acid was destroyed by ascorbic acid oxidase and heat treatments.

the rate of ascorbic acid oxidation. Some ascorbic acid remained after four days in some samples (e.g., no. 18), but none was present in others (e.g., no. 17). Apparently, part of the hydrogen peroxide was used in oxidizing compounds other than ascorbic acid in some milks, because there was residual ascorbic acid even though two moles of hydrogen peroxide were added per mole of ascorbic acid. Lipid peroxidation occurred in the samples with residual ascorbic acid.

There was an increase in TBA absorbancy in one sample (no. 17) to which hydrogen peroxide was added after destruction of ascorbic acid by ascorbic acid oxidase. Oxidized flavor was not detected in any samples subjected to this treatment.

Fig. 8 compares ascorbic acid oxidation curves for four untreated milks (controls) with samples in which ascorbic acid was destroyed by hydrogen peroxide ($1.76 \times 10^{-3} M$) and to which L-ascorbic acid was re-added. Two of the milks changed their susceptibility classification as a result of this treatment. No. 14 changed from spontaneous to susceptible, and no. 4 from susceptible to spontaneous, whereas nos. 10 and 11 were spontaneous before and after the depletion-addition treatment. The milks differed markedly in the influence of the treatment on the ascorbic acid oxidation curves. The results obtained were inadequate to explain the differences, which could have been caused by changes in antioxidant concentration, copper binding, or other variables.

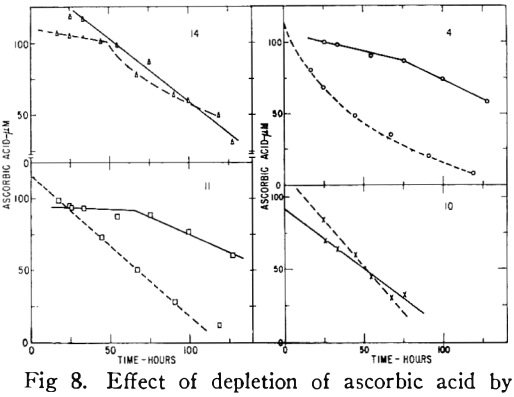


Fig 8. Effect of depletion of ascorbic acid by hydrogen peroxide ($1.76 \times 10^{-3} M$) on subsequent oxidation of re-added L-ascorbic acid at 3–4°C in milks of 4 cows. Solid line, controls; broken line, after depletion and addition.

Results of experiments such as these emphasize that studies of the mechanism of oxidized flavor in milk based on treatments with hydrogen peroxide could be misleading because of the varied changes that hydrogen peroxide may produce. However, they indicate that hydrogen peroxide is not the initiator of lipid peroxidation in milk.

The following criticisms are made of earlier evidence in support of the hypothesis that hydrogen peroxide initiates lipid peroxidation in milk. Olson and Brown (1942), and Brown and Olson (1942) added hydrogen peroxide at levels greatly in excess of

those that might be produced by oxidation of the ascorbic acid present in milk. From 2.9×10^{-3} to $2.9 \times 10^{-4} \mu M$ hydrogen peroxide (0.01 to 0.1%, presumably of 1% solution) were added to washed milk. The ascorbic acid in milk (20 mg/L or $114 \mu M$) could not produce more than $114 \mu M$ of hydrogen peroxide. Even at the high levels of hydrogen peroxide added to washed milk, no oxidized flavor was produced unless copper (0.5 to 1.5 $\mu g/g$) was also added. Furthermore, Olson and Brown (1942) produced oxidized flavor in washed milk by adding ascorbic acid without added copper. Ottolenghi (1959) also showed that the continuous addition of hydrogen peroxide or the presence of systems that generate hydrogen peroxide, e.g., xanthine oxidase, had no effect on the peroxidation of mitochondrial lipids.

The ascorbic acid radical in lipid peroxidation. It has been postulated that the ascorbic acid radical is the peroxidation initiator in milk (King, 1958) and some other materials (Kelly and Watts, 1957). Yamazaki and Piette (1961) demonstrated the presence of ascorbic acid radical when oxidation of ascorbic acid is catalyzed by ascorbic acid oxidase, and found that the concentration of the radical increased with the concentration of the enzyme. In the experiment described below, ascorbic acid oxidase was used to study the role of ascorbic acid radical in lipid peroxidation in milk.

Ascorbic acid in four milks was destroyed by ascorbic acid oxidase and heat treatment, and D- and L-ascorbic acid was added ($114 \mu M$) to separate portions. The rate of oxidation of the re-added ascorbic acid was determined, as were changes in TBA absorbancy and flavor score. It was assumed that ascorbic acid radical concentration was proportional to the rate of ascorbic acid oxidation, and that, in the reaction below, equilibrium was far to the right.

$$R + T \rightleftharpoons 2S$$

where R = reduced ascorbic acid, T = oxidized ascorbic acid, and S = radical (or semiquinone).

As shown in Table 2, the rate of ascorbic acid oxidation catalyzed by ascorbic acid oxidase with either re-added L- or D-ascorbic acid was much greater than in the control

Table 2. Effect of ascorbic acid radical concentration,[a] produced by ascorbic acid oxidase, on lipid peroxidation (3–4°C).

Sample	Rate [b] of ascorbic acid oxidation (μM per hr $\times 10^2$)	TBA increase at 5 days (absorbancy $\times 10^3$)	Flavor score at 5 days
17 Control	98	120	4
AA destroyed [c]	0	−2	0
L-AA re-added [d]	240 [e]	108	4
D-AA re-added [d]	194	114	4
18 Control	53	5	0
AA destroyed	0	3	0
L-AA re-added	213 [e]	14	0
D-AA re-added	156	28	3.2
19 Control	27	−2	0
AA destroyed	0	0	0
L-AA re-added	142	9	1.8
D-AA re-added	83	2	2
20 Control	31	1	0
AA destroyed	0	1	0
L-AA re-added	117	8	0
D-AA re-added	82	15	2

[a] Assumed that radical concentration was proportional to rate of ascorbic acid oxidation.
[b] Initial rates. The reaction slowed with time.
[c] By ascorbic acid oxidase and heat treatments.
[d] $114 \mu M$ (20 mg/L).
[e] Ascorbic acid completely oxidized in two days.

samples. Lipid oxidation and rate of ascorbic acid oxidation showed no consistent relation such as would have been expected if the ascorbic acid radical produced by ascorbic acid oxidase catalysis initiated the lipid peroxidation. These results do not support the hypothesis that the ascorbic acid radical initiates lipid peroxidation in milk.

Furthermore, King (1958) inhibited lipid peroxidation with EDTA (ethylenediaminetetraacetic acid), but ascorbic acid oxidation was enhanced under the same conditions. Yamazaki *et al.* (1961) have shown that the ascorbic acid radical is produced in the presence of Cu-EDTA and Fe-EDTA.

REFERENCES

Allan, J. E. 1950. The oxidation of ascorbic acid in dairy products. *J. Dairy Research* **17**, 54.

Brown, W. C., and F. C. Olson. 1942. Oxidized flavor in milk. XII. Further studies of ascorbic acid mechanisms in the production of oxidized flavor in milk. *J. Dairy Sci.* **25**, 1041.

Castelfranco, P. 1960. Inhibition of some metalloprotein enzymes by 3-amino-1,2,4-triazole. *Biochim. et Biophys. Acta* **41**, 485.

Dawson, C. R., and R. J. Magee. 1955. Ascorbic acid oxidase. "Methods in Enzymology II." p. 831. (S. P. Colowick and N. O. Kaplan, eds.) Academic Press, N.Y.

Dunkley, W. L., and W. G. Jennings. 1951. A procedure for application of the thiobarbituric acid test to milk. *J. Dairy Sci.* **34**, 1064.

Frieden, E., and I. W. Maggiolo. 1957. Activation and other properties of ascorbic acid oxidase. *Biochim. et Biophys. Acta* **24**, 42.

Greenbank, G. R. 1940. Variation in the oxidation-reduction potential as a cause for the oxidized flavor in milk. *J. Dairy Sci.* **23**, 725.

Greenberg, L. D. 1957. Water-soluble vitamins. II. *Ann. Rev. Biochem.* **26**, 209.

Holmes, A. D. 1952. Ascorbic acid in tocopherol-enriched milk. Food Research **17**, 367.

Kelly, G. G., and B. M. Watts. 1957. Effect of copper chelating agents on the prooxidant activity of ascorbic acid with unsaturated fats. *Food Research* **22**, 308.

King, R. L. 1958. Variation and distribution of copper in milk in relation to oxidized flavor. Ph.D. thesis, Univ. of Calif. Library, Davis, Calif.

King, R. L., J. R. Luick, I. I. Litman, W. G. Jennings, and W. L. Dunkley. 1959. Distribution of natural and added copper and iron in milk. *J. Dairy Sci.* **42**, 780.

Krukovsky, V. N. 1949. Ascorbic acid oxidation in milk by preformed hydrogen peroxide. *J. Dairy Sci.* **32**, 163.

Krukovsky, V. N., and E. S. Guthrie. 1945. Ascorbic acid oxidation a key factor in the inhibition or promotion of the tallowy flavor in milk. *J. Dairy Sci.* **28**, 565.

Krukovsky, V. N., and E. S. Guthrie. 1946. Vitamin C, hydrogen peroxide, copper and the tallowy flavor in milk. *J. Dairy Sci.* **29**, 293.

Olson, F. C., and W. C. Brown. 1942. Oxidized flavor in milk. XI. Ascorbic acid, glutathione, and hydrogen peroxide as mechanisms for the production of oxidized flavor. *J. Dairy Sci.* **25**, 1027.

Ottolenghi, A. 1959. Interaction of ascorbic acid and mitochondrial lipids. *Arch. Biochem. Biophys.* **79**, 355.

Riel, R. R., and H. H. Sommer. 1954. Oxidized flavor in milk. I. Review of literature. Contribution de L'institut D'Oka (Université de Montreal) La Trappe, P.Q., Canada, No. 10.

Sharp, P. F. 1938. Rapid method for the quantitative determination of reduced ascorbic acid in milk. *J. Dairy Sci.* **21**, 85.

Smith, G. J., and W. L. Dunkley. 1961. Prooxidants in spontaneous development of oxidized flavor. *J. Dairy Sci.* (in press).

Snedecor, G. W. 1956. "Statistical Methods." 5th ed. Iowa State College Press. Ames.

Stewart, A. P., and P. F. Sharp. 1945. Determination of vitamin C in the presence of interfering reducing substances. *Ind. Eng. Chem. Anal. Ed.* **17**, 373.

Strobel, D. R., W. G. Bryan, and C. J. Babcock. 1953. Flavors in milk: a review of literature. U. S. Dept. Agr., Washington.

Swanson, A. M., and H. H. Sommer. 1940. Oxidized flavor in milk. I. Effect of the development of oxidized flavor on the iodine number of the phospholipid fraction of milk. *J. Dairy Sci.* **23**, 201.

Timberlake, G. F. 1960. Metallic components of fruit juices. III. Oxidation and stability of ascorbic acid in model systems resembling blackcurrant juice. *J. Sci. Food Agr.* **11**, 258.

Yamazaki, I., and L. H. Piette. 1961. Mechanism of free radical formation and disappearance during ascorbic acid oxidase and peroxidase reaction. *Biochim. et Biophys. Acta.* (in press).

Yamazaki, I., H. S. Mason, and L. H. Piette. 1961. *J. Biol. Chem.* (in press).

Extraction of Lipids from Oxidizing Mullet[a]

MARELYNN W. ZIPSER, JACQUELINE DUPONT, AND BETTY M. WATTS
Department of Food and Nutrition, Florida State University, Tallahassee

(Manuscript received October 23, 1961)

SUMMARY

Total lipids, lipid phosphorus, and total polyenes extracted from mullet tissues with chloroform-methanol show a progressive decrease in oxidizing mullet tissue. Thiobarbituric acid values from chloroform-methanol extracts of oxidized tissues are approx 30–50% of those obtained from the unextracted tissue. Much of the TBA-reactive material becomes concentrated in the interfacial fluff during the washing of such extracts. Chloroform alone extracts only about 10% of the TBA-reactive material.

Numerous observations in this laboratory have established that lipid oxidation as measured by the 2-thiobarbituric acid (TBA) test and organoleptic panels was very rapid in the cooked flesh of meat animals and fin fish. To demonstrate this oxidation it was necessary to apply the TBA test to distillates of the whole tissue (Tarladgis *et al.*, 1960). Chloroform extracts of meats generally showed little or no increase in TBA numbers under conditions where the TBA number of the whole tissue increased 10–50-fold. It was hypothesized that where such discrepancies occurred, the lipid fractions mainly involved in the oxidation were phospholipids or proteolipids not extracted with chloroform (Younathan and Watts, 1960).

Folch *et al.* (1951, 1957) and Folch and Lees (1951) have described extraction procedures for the quantitative removal of total lipids from animal tissues. Their procedures, involving the use of chloroform-methanol as solvents, were demonstrated to remove phospholipids and proteolipids, as well as triglycerides, from various fresh tissues of land animals.

It has been demonstrated in this laboratory that most of the oxidizable lipid from raw fish tissue is removed by the Folch procedure. However, preliminary observation indicated that chloroform-methanol did not remove all of the TBA-reactive material from cooked, oxidizing fish tissue.

This observation led to the present, more systematic, study of the lipids extracted from a fin fish during oxidation. It was considered probable that peroxidation or oxidative scission of fatty acid components decreased lipid solubility in fat solvents. This might be expected to show up as a progressive decrease in lipid extracted from oxidizing tissues as well as in low TBA numbers of lipid extracts. These predictions were tested in the following experimental work.

MATERIALS AND METHODS

Preparation of fish. Mullet (*Mugil cephalus*) was used throughout this study. The fresh fish were filleted, skinned, and trimmed at a local market. The fillets for any one experiment were ground in an electric food chopper and mixed thoroughly by hand to obtain a homogeneous product.

The mullet has a fairly well defined lateral line band of darker tissue. In several experiments this tissue was separated as completely as possible in the raw fish with a knife, and the light and dark meat were ground and cooked separately. It has been established that the rate of lipid oxidation differs greatly in cooked light and dark tissue. The intensity of the reaction is greater in dark tissue, where a large quantity of highly unsaturated lipids and ferric heme pigments, thought to be catalysts for lipid oxidation, are located (Zipser and Watts, 1961).

In several experiments an antioxidant was used on a portion of the fish tissue. This consisted of 0.5% sodium tripolyphosphate and 0.1% sodium

[a] Supported in part by a grant, RG-6885, from the National Institutes of Health, U. S. Public Health Service.

ascorbate. The antioxidants were mixed thoroughly by hand into the raw ground fish.

Cooking and storage. Approximately 175-g portions of ground fish were packed in 307 × 113 C-enameled cans, which were sealed and placed in a boiling water bath and cooked to an internal temperature of 70°C. This required approximately 20 minutes. The cans were then cooled in a running-water bath, and opened, and the cooked fish was removed, mixed thoroughly with a fork, and stored in covered bowls in a refrigerator.

Lipid extraction. Three methods were used for lipid extraction. The method described by Folch *et al.* (1951) involves homogenization of fish tissue with a chloroform-methanol solution, filtration to obtain a clear extract, and washing to remove non-lipid contaminants. The last step is accomplished by pipetting suitable aliquots of the filtrate into a small beaker at the bottom of a large beaker of distilled water. After standing overnight there results an upper transparent water-methanol phase, a lower chloroform phase, and an accumulation of material at the interface. The water-methanol phase is removed by suction, and the surface is washed with distilled water. Next, the chloroform portion is removed by pipette and saved for further testing. The lower area is washed with chloroform, and the washings added to the chloroform extract. Finally, the fluffy material is quantitatively transferred to a volumetric flask with methanol.

It was discovered that the lipid components could undergo further oxidation in the washing process. This could be effectively eliminated by carrying out the overnight washing in tall 1-L beakers and using recently boiled and cooled glass-distilled water for the separation. No significant differences were found in the amount of fluff separated at 25 and 5°C.

Folch *et al.* (1957) described a modification of the original method in which 0.2 volume of a water solution of various salts was added to the extract, and two phases separated. This prevented separation of proteolipids at the interface and gave a clear solution of total lipids in the chloroform phase. The salt solutions employed in our experiments were 0.58% NaCl and 0.88% KCl.

Chloroform extracts, used for comparison with the Folch extract in one experiment, were prepared as described by Erdman *et al.* (1954).

Table 1. Lipid recovery from raw mullet tissues.

	Lipid recovered [a]			
	Folch method (1951)			Folch (1957)
Sample	Chloroform	Fluff	Total	Total extract
Dark meat	6.00	0.55	6.55	6.65
Light meat	1.45	0.28	1.73	1.74

[a] As % of fresh tissue.

Polyenes. Total polyunsaturated fatty acids were estimated by converting to the conjugated products with an excess of lipoxidase according to the method of MacGee (1959). The factor used by MacGee to convert optical density of the conjugated fatty acids to percent polyenes in the tissue was derived for vegetable fatty acids, which may differ from fish lipids in chain length. However, in the absence of data on the mean molecular weight of the fatty acids from mullet, the same factor was used. The percentages obtained can be considered only as approximations.

Lipid phosphorus. The micromethod of Chen *et al.* (1956) was used for phosphorus determinations, employing a Kjeldahl micro apparatus for the digestions. A 10:1:1 mixture of HNO_3, H_2SO_4, and $HClO_4$ was used rather than adding each acid individually.

TBA. The distillation method described by Tarladgis *et al.* (1960) was used. When applied to lipid extracts the solvents were first removed at 65°C in a stream of nitrogen.

RESULTS

Weight of lipid extracted. Preliminary extractions of the same samples of mullet tissue gave similar results for the two procedures described by Folch. That is, the total lipids obtained by preventing the formation of interfacial fluff were approximately the same as the sum of the chloroform and fluff fractions (Table 1).

Table 2 shows the changes in total lipid and lipid phosphorus obtained upon cooking dark meat of mullet and storing the cooked material in the refrigerator for 5 days. A 21% decrease in total

Table 2. Total lipids and lipid phosphorus in oxidizing dark meat.

Sample	Total lipids (% of tissue)	Lipid phosphorus (% of tissue)	TBA number (mg malon./1000 g tissue)
Raw (0 days)	8.22	.0370	
Cooked			
0 days	7.64	.0387	3.2
3 days	6.85	.0332	60
5 days	6.52	.0315	86

Table 3. Polyenes extracted from oxidizing mullet.

Sample	Days of storage	Expt. 1—Dark meat TBA number	Expt. 1—Dark meat Polyenes (% of tissue)	Expt. 2—Whole mullet TBA number	Expt. 2—Whole mullet Polyenes (% of tissue)
Raw	0	16	3.58	1.4	0.36
Cooked (no antiox)	0	11	3.30	1.8	0.39
	3	106	3.10	15	0.29
	6	164	2.92	22	0.32
	9	....	....	25	0.30
Cooked (antiox)	0	7.3	3.53	0.8	0.40
	3	9.5	3.36	0.9	0.41
	6	14	3.31	0.4	0.40
	9	....	....	0.4	0.42

lipids and a 15% decrease in lipid phosphorus occurred during the five days of refrigerated storage. As shown by the increase in TBA number, oxidation was very rapid during this storage period. In another similar experiment, the decrease in total lipids was 13%, and in phosphorus 18%.

Table 3 shows loss of polyenes during storage of two samples of cooked mullet tissue. In the first experiment, with dark meat only, the antioxidant protected the sample only partially, but the protection is reflected in higher retention of polyenes. The fish used in the second experiment on whole mullet was very low in fat, so the changes in polyenes are somewhat less reliable. However, a significant drop occurred in the polyenes of the unprotected sample. Oxidation was completely inhibited by the antioxidant, with no loss of polyenes.

TBA values of extracted lipids. A comparison was made of the TBA values obtained on whole mullet tissue and on total lipids (Folch *et al.*, 1951) and chloroform extracts (Erdman *et al.*, 1954) of the same tissue. The mullet tissue was cooked and exposed in the refrigerator over a 12-day period with extractions at daily intervals.

The results are shown in Fig. 1. The very low and irregular values obtained from the chloroform extracts are particularly noteworthy. The chloroform would not, of course, be expected to extract phospholipids or proteolipids, but it is doubtful that the very low TBA values are to be interpreted as evidence that triglycerides are not oxidizing. Again it seems more probable that a large fraction of the oxidized triglyceride is not extracted by chloroform.

In this experiment the interfacial fluff was not separated, and the lipid extract was not washed further after standing overnight in contact with the aqueous phase, so that the weight of dried material obtained from the extracts may be somewhat greater than the total amount of lipid. The weight

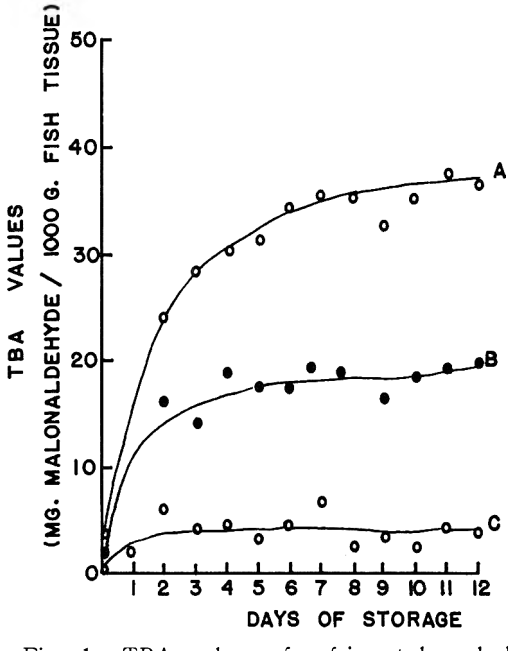


Fig. 1. TBA values of refrigerated cooked muscle tissue as compared to those of lipid extracts from the same tissue. A) whole mullet; B) chloroform-methanol extract; C) chloroform extract.

of dried material from the chloroform-methanol extracts was 7.5% of the whole mullet tissue, whereas the fraction extracted by chloroform alone was 5.1%. If the observed TBA numbers are calculated as mg. malonaldehyde per 1000 g lipid at this storage period, the respective TBA numbers obtained from the lipid in the unextracted tisue, the chloroform-methanol extract, and the chloroform extract are 470, 243, and 80.

A second experiment was designed to determine how much of the TBA-reactive material is in the fluff. A chloroform-methanol extract was

Table 4. TBA values of lipid fractions from cooked, whole mullet tissue.

Lipid fraction	Weight of lipid g/100 g tissue	Malonaldehyde after storage [a]	
		(mg/1000 g whole tissue)	(mg/1000 g lipid fraction)
Folch extract			
Chloroform fraction	4.54	6.0	132
Interfacial fluff	0.33	5.7	1900
Unextracted tissue	4.87[b]	36	740

[a] Stored in the refrigerator for 10 days.
[b] Assumed to be the sum of the two fractions above.

prepared from cooked whole mullet tissue that had been stored in the refrigerator for 10 days. Washed interfacial fluff and chloroform fractions were dried to determine the weight of lipid, and similar extracts from other portions of the same tissue were used for TBA values.

The results (Table 4) bring out several points of interest. As in the previous experiment, the TBA values from all of the lipid extracted with chloroform-methanol (sum of chloroform and fluff fractions) are still much lower than the values obtained on the unextracted tissue. The TBA number of the fluff, calculated to the weight of dried material, is extremely high, considerably higher than the maximum TBA values recorded in this laboratory during the accelerated oxidation of pure, unsaturated fatty acids catalyzed by heme compounds or lipoxidase. It seems probable that TBA-reactive fatty acid fragments tend to accumulate at the interface, possibly bound to proteins.

DISCUSSION

The evidence demonstrates the pitfalls to be expected when one attempts to estimate the extent of lipid oxidation in animal tissues by performing tests for the oxidation products on lipid extracts from the tissue. Even if the extraction procedure used is known to remove substantially all of the lipid material from fish tissue in the cold, and care is exercised to prevent further oxidation during the extraction itself, changes in the solubility of the oxidized lipid material interfere drastically with its extraction and estimation. Extracts obtained with nonpolar solvents such as chloroform or carbon tetrachloride would appear to be of very little use in studies of lipid oxidation in muscle tissue.

The three tests used to follow lipid changes (total lipids, lipid phosphorus, and polyenes) show losses of approximately the same magnitude. Although the oxidation undoubtedly occurs mainly in the polyenes, their oxidation could affect the solubility of the larger triglyceride or phospholipid molecules of which they are a part. The fatty acids of the triglycerides of fish tisues do not differ greatly in degree of unsaturation from those of the phospholipids.

REFERENCES

Chen, P. S., Jr., T. Y. Toribara, and H. Warner. 1956. Micro-determination of phosphorus. *Anal. Chem.* **28**, 1756.

Erdman, A. M., B. M. Watts, and L. C. Ellias. 1954. Smoke flavor and ascorbic acid as preservatives for fatty fish. *Food Technol.* **8**, 320.

Folch, J., and M. Lees. 1951. Proteolipides, a new type of tissue lipoproteins: Their isolation from brain. *J. Biol. Chem.* **191**, 807.

Folch, J., I. Ascoli, M. Lees, J. Meath, and F. LeBaron. 1951. Preparation of lipide extracts from brain tissue. *J. Biol. Chem.* **191**, 833.

Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226**, 497.

MacGee, J. 1959. Enzymatic determination of polyunsaturated fatty acids. *Anal. Chem.* **31**, 298.

Tarladgis, B. G., B. M. Watts, M. T. Younathan, and L. Dugan. 1960. A distillation method for the quantitative determination of malonaldehyde in rancid foods. *J. Am. Oil Chemists' Soc.* **37**, 44.

Younathan, M. T., and B. M. Watts. 1960. Oxidation of tissue lipids in cooked pork. *Food Research* **25**, 538.

Zipser, M. W., and B. M. Watts. 1961. Oxidative rancidity in cooked mullet. *Food Technol.* **15**, 318.

The Effect of Storage at Elevated Temperature on Some Proteins of Freeze-Dried Beef[a]

L. J. N. COLE

Defence Research Medical Laboratories, Toronto 12, Ontario

(Manuscript received November 9, 1961)

SUMMARY

In an attempt to determine the effect of oxygen and elevated temperature on freeze-dried beef in a moisture-free atmosphere, two series of storage experiments were conducted on freeze-dried beef slices. In one longissimus dorsi muscle, and in the other semitendinosus, was packaged under commercially feasible conditions where the residual gas contained 2.5 ± 0.5% oxygen and no atmospheric moisture. An in-can desiccant, calcium oxide (CaO), and an oxygen scavenger, Oxyban (glucose oxidase), were added in some instances, and the cans were stored up to 6 months at 100°F.

In each series there was marked loss in extractability of actomyosin, and in activity of actomyosin ATPase after 1 month, but only a 40% loss of sarcoplasmic proteins. The residual aldolase activity decreased progressively to 8% at 6 months, whereas the residual water-soluble proteins decreased progressively only to 56%. There was a marked difference between the two series in percent rehydration at 6 months: 81.1 in one, and only 26 in the second. Electrophoresis of the sarcoplasmic proteins showed gross denaturation after 1 month at 100° F.

INTRODUCTION

The three main environmental factors affecting the stability of freeze-dried beef are moisture, oxygen, and temperature. This investigation was designed to study the effect of storage at elevated temperature on freeze-dried beef in the presence of small quantities of oxygen, such as would be present in a commercial packaging operation, i.e., about 2.5±0.5%. Uncooked freeze-dried beef deteriorates rapidly when left exposed to air at room temperature (Harper and Tappel, 1957). It has been demonstrated that, under these conditions, the rate of oxygen uptake by the product is greater than can be accounted for by the amount of oxygen required to oxidize the heme proteins (myoglobin and hemoglobin), the compounds usually considered to be most susceptible to oxidation. Tappel (1956) reports also that potentially oxidizable groups such as sulfhydryl are not present in sufficient amount to account for the total uptake of oxygen. It has been suggested, therefore, that there is direct oxygen uptake by the contractile protein, although the mechanism by which this would occur is in doubt.

The deleterious effect of moisture on acceptability and on adenosine triphosphatase activity has been demonstrated by Hunt and Matheson (1959), but there are few reports on the biochemical changes resulting from storage. We are primarily interested in ascertaining whether two enzymes, each representative of a different group of proteins, were affected equally by storage, and what effect storage had on the "solubility" of various components.

EXPERIMENTAL

In every instance the following basic canning procedure was followed. Freeze-dried slices taken from the drying chamber (Smithies and Blakley, 1959) were put immediately into cans along with desiccant, calcium oxide (CaO), and an oxygen scavenger, Oxyban, where applicable, and the can

[a] DRML Report No. 232-8; PCC Project No. D50-78-03-01. Paper presented at the 21st annual meeting of the Institute of Food Technologists, May, 1961, New York.

was closed under 25 in. Hg with an "American Can" closing machine. The cans were then punctured and placed in a chamber, re-evacuated, and flushed with nitrogen. Since it was impossible to estimate the amount of residual oxygen in cans containing freeze-dried beef before storage, a group of 2 dozen cans without inclusion was put through the above procedure. Residual oxygen was measured by the method of Dale (1957) modified for application to cans. The average value was $2.5 \pm 0.5\%$ residual atmospheric oxygen.

Two series of storage experiments were conducted. In series A, using semitendinosus, an attempt was made to compare the effect of calcium oxide (CaO) alone and of Oxyban + CaO with that of nitrogen packing only. Series B was conducted on longissimus dorsi muscle, all cans containing CaO and Oxyban.

Water extract. An amount of freeze-dried beef equivalent to 2.0 g wet weight was cut into very small pieces and minced in a Virtis blender with 15 ml of water. The entire mash was poured into 40-ml centrifuge tubes and spun 15 min in a clinical centrifuge at 1500 G. The supernatant liquid was decanted, and the pellet re-extracted with 15 ml water by macerating with a glass rod. The spinning was repeated and the supernatant liquids were combined. This was labeled "water extract."

Sarcoplasmic proteins. Freeze-dried beef (1.0 g), equivalent to 3.5 g wet weight, was cut into small pieces and blended with 25 ml of phosphate buffer solution of 0.15 ionic strength and pH 7.6 (Bosch, 1951). In all, 3 extractions (25 ml each) were made in a manner similar to that described in the preceding paragraph, and the supernatant liquids combined. This was then filtered through Whatman No. 12 paper, and the filtrate was used for analysis of sarcoplasmic proteins.

Fibrils. The fibril fraction was prepared according to the method of Perry (1953).

Actomyosin. The freeze-dried equivalent of 3.5 g fresh or frozen beef was extracted with potassium chloride-sodium bicarbonate solution of 0.53 ionic strength at pH 8.25 in a manner previously described (Cole and Smithies, 1960). A suitable portion, usually 20 ml for control samples and 40 ml for stored samples, was diluted with 100 or 200 ml of water, respectively, and allowed to stand overnight. The mixture was then spun for 30 min at 1500 G, the supernatant liquid discarded, and the pellet taken up in 20 ml or 10 ml, respectively, of buffer of 0.53 ionic strength. In order to obtain an amount of extract from stored samples sufficient for analysis it was necessary to reduce the volume of buffer in the final solution.

Adenosine triphosphatase (ATPase) activity. The final concentration of reactants was ATP, $2.5 \times 10^{-3}M$ (disodium salt, sigma); calcium chloride, $5 \times 10^{-3}M$; potassium chloride $10^{-1}M$; tris-(hydroxymethyl aminomethane), $5 \times 10^{-2}M$; muscle extract, 0.2–0.3 mg N.

The reaction was carried out at pH 8.2 in the manner previously described (Cole and Smithies, 1960). Tubes were centrifuged and suitable aliquots taken for free phosphorus analysis, which was determined by the classical Fiske and Subbarow method except that "Elon" (mono-methyl-*p*-aminophenol sulfate) was used for color development instead of aminonaphthol sulfonic acid.

2,4-Dinitrophenol. Recrystallized 2,4-dinitrophenol (DNP) was dissolved in ethyl alcohol and kept as a stock solution of $4 \times 10^{-2}M$. The final concentration of DNP in the incubation mixture was $4 \times 10^{-3}M$.

Aldolase activity. Aldolase activity was determined by the procedure of Taylor *et al.* (1948). The effective concentration of water extract that gave optimum activity was found to be between 0.0008 and 0.012 mg N under our conditions. There appeared to be a shift in optimum between fresh, frozen, and freeze-dried beef, and this shift was assumed to indicate that, with processing, there was a decrease in the ratio of effective enzyme nitrogen to total nitrogen. In order to obtain the optimum activity in instances where optimum nitrogen concentration was in doubt, three levels of extract nitrogen were used, and the one giving the highest activity was accepted.

Rehydration ratio. Freeze-dried beef of known weight (*ca.* 5 g) was immersed 5 min in water, removed, dried on filter paper (½ min each side), and reweighed. The amount of water taken up per gram dry weight is called the rehydration ratio. The value given in the tables as percent rehydration is a comparison of the rehydration ratios of stored and control samples, expressed as a percentage of the latter. The rehydration of stored freeze-dried beef is a time-dependent function that can be used as an index of deterioration. In our experience, stored products can be made to take up their original weight of water if soaked for prolonged periods (30 min to 1 hr). The 5-min rehydration time was arbitrarily chosen as one that was long enough to enable control samples to take up their original amount of water, and short enough to give values that reflect changes in stored products.

Electrophoresis. Electrophoresis was carried out in a Perkin-Elmer apparatus, model 38A. The extract was dialyzed against an $Na_4P_2O_7$–HCl buffer, I = 0.13, pH 8.6, for 52 hours, and subjected to electrophoresis at 4°C and 1.46 watts.

Nitrogen was determined by the standard micro-Kjeldahl procedure.

RESULTS AND DISCUSSION

The sarcoplasmic group of proteins contain, in addition to myoglobin and hemoglobin, all the enzymes of the glycolysis and citric acid cycles, most of these fractions being present in the water extract. Aldolase was chosen as representative of the enzymes of glycolysis. It is realized that results obtained for this enzyme cannot be taken as necessarily indicative of the behavior of the other enzymes and protein fractions. Nevertheless, the intent was to compare one member of the sarcoplasmic group, which apparently withstands storage well (Table 1), with the ATPase of the contractile group, which deteriorates rapidly during storage.

From *a priori* reasoning it was thought that freeze-dried beef protected by CaO and Oxyban should deteriorate less rapidly than samples packaged with nitrogen only. Consequently, in series A, done first, samples protected by CaO and Oxyban were not analyzed until after 3 months, whereas those with nitrogen only were analyzed at 1, 2, 3, and 5 months. In series B all cans contained CaO and Oxyban.

From the results shown in Tables 1 and 2, solubility data demonstrate that the sarcoplasmic proteins (including water extractables) are much less adversely affected than those of the contractile group. A total of 97.5% and 85%, respectively, of extractable sarcoplasmic proteins was recovered after 1 month of storage, whereas only 3% and 8% of the originally "soluble" contractile proteins could be extracted. A similar situation exists in the case of the residual aldolase activity of the water extract and the ATPase of the actomyosin extracts (Table 3). Whereas 40.6% of the aldolase activity remained after 1 month, ATPase activity had fallen to 13% of the original value in this period. Further, there is a gradual reduction in aldolase activity from 40.6 to 8%, but the loss of ATPase activity is sudden and complete between 1 and 2 months.

When the sarcoplasmic extract from the sample stored for 1 month was subjected to electrophoresis, gross alteration of the pattern was found when compared with the control (Figs. 1 and 2). It is very difficult under these circumstances to make a quantitative estimate (from the electrophoretic pattern) of the amount of destruction suffered by the components. The interesting feature is that, despite these changes, 40% of the original aldolase activity was still elicited.

The rehydration ratio done under these conditions showed great variability between the two series. Since there was strict control of two of the three variables, *viz,* moisture and temperature, it would appear that the variability in rehydration ratio can be ascribed, at least in part, to variability in residual oxygen concentration. Furthermore, there appears to be no relationship between the ability of the product to rehydrate and the extractability of the contractile protein.

Table 4 shows the ATPase activity of myofibrils obtained from control and stored samples. The loss in activity in 1 month of storage is 50%, and a slow, erratic decline

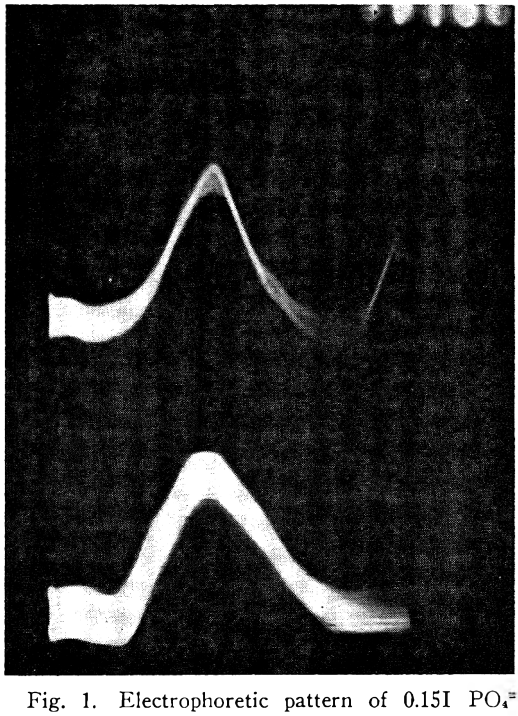


Fig. 1. Electrophoretic pattern of 0.15I $PO_4^{=}$ extract of stored freeze-dried beef dialyzed vs. 0.13I $Na_4P_2O_7$–HCl pH 8.6. Voltage 162, current 9.0 milliamp.

Time descending ⟵ 5100 sec
(upper)
ascending ⟶ 5160 sec

Table 1. Analysis of freeze-dried beef stored at 100°F (Series A).

Duration of storage	Storage Condition	Sarcoplasmic proteins (% recovered)	Actomyosin (% recovered)	% rehydration (5 min)
1 month	N = nitrogen only	97.5	3.0	98.0
2 months	N	88.1	2.3	60.2
	N + CaO	88.9	2.3	92.0
3 months	N	81.6	5.4	85.2
	CaO + Oxyban + N	82.8	7.0	84.4
5 months	N only	66.5	2.45	52.0
6 months	N + CaO	60.2	2.8	86.2
	CaO + Oxyban + N	67.0	2.3	81.1
Control (after 8 months at −20°F)	N_2 only	98.6	91.3	98.6

Table 2. Analysis of freeze-dried beef stored at 100°F (Series B).[a]

Duration of storage	Water extract (% N recovered)	0.15 μ extract sarcoplasmic (% N recovered)	0.53 μ extract (actomyosin) (% N recovered)
1 month	86.0	85.0	8.0
2 months	71.0	70.0	6.5
3 months	53.0	65.0	6.1
4 months	57.0	75.0	4.7
5 months	63.0	74.0	3.5
6 months	56.0	64.0	2.9
Control (−20°F) 6 months	100	100	100

[a] All cans contained CaO and Oxyban.

Table 3. Analysis of freeze-dried beef stored at 100°F (Series B).[a]

Duration of storage	Aldolase % activity recovered	Actomyosin ATPase (μg P/min/mg N)		% rehydration (5 min)
		No DNP	% stimulation	
1 month	40.6	7	25	93.2
2 months	23.8	....	....	66.2
3 months	25.9	....	....	31.4
4 months	16.2	....	....	13.3
5 months	15.0	....	....	28.7
6 months	8.0	....	....	26.2
Control (−20°F)	100	52[b]	30[b]	100

[a] All cans contained CaO and Oxyban.

[b] These are initial ATPase values. On storage for 6 months at −20°F there is no significant change in ATPase, but DNP no longer stimulates the reaction.

in activity is observed during the remainder of the storage period. This behavior is in contrast with that of the actomyosin ATPase. This evidence indicates that there is probably a difference in the behavior of Ca++-activated myofibrillar ATPase and actomyosin ATPase, a circumstance that might be expected in the light of previous work (Perry and Chappell, 1957). Since it was intended, in future work, to compare the "in vacuo" storage characteristics at 100°F of fibrils *per se,* ATPase determinations were carried out on fibrils prepared from these stored products, and the values are reported here.

The data reported show that biochemical damage to protein resulting from storage deterioration can be measured. The concentration of residual atmospheric oxygen consonant with good storage characteristic is low and appears to be critical at this temperature, even in the absence of atmospheric moisture. However, it has been our experience that although there is profound biochemical damage, the product made in these laboratories does not become unacceptable to a taste panel until after 6 months, provided the freeze-drief beef is soaked long enough.

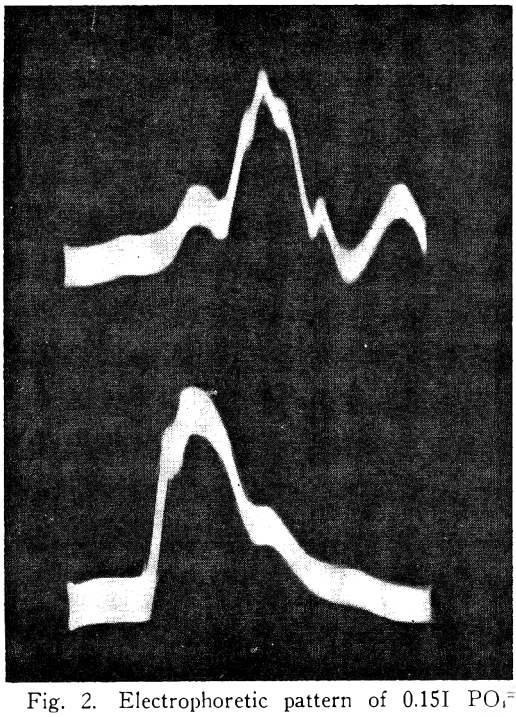


Fig. 2. Electrophoretic pattern of 0.15I $PO_4^{=}$ extract of freshly prepared freeze-dried dialyzed vs. 0.13I $Na_4P_2O_7$–HCl pH 8.6. Voltage 162, current 9.0 milliamp.

Time descending ⟵ 9650 sec
(upper)
ascending ⟶ 9710 sec

Table 4. Adenosine triphosphatase activity of fibrils from freeze-dried beef stored at 100°F (Series B).

Duration of storage	ATPase activity μg P/min/mg N	
	Without DNP	+DNP $4\times10^{-3}M$
1 month	11.0	11.0
2 months	7.6	4.1
3 months	4.7	3.7
4 months	6.2	4.0
5 months	6.7	4.7
6 months	....	....
Control, 6 months at −20°F	22.8	30.6

ACKNOWLEDGMENT

The author acknowledges the technical assistance of Mrs. Helen Kent.

REFERENCES

Bosch, M. W. 1951. Quantitative electrophoretic study of rabbit muscle protein soluble in dilute salt solutions. *Biochim. et Biophys. Acta.* **7**, 61.

Cole, L. J. N., and W. R. Smithies. 1960. Methods of evaluating freeze-dried beef. *Food Research* **25**, 363.

Dale, Robert F. 1957. Simplified micro-gas analysis. *Modern Packaging* 30, 150.

Harper, J. C., and A. L. Tappel. 1957. Freeze-drying of food products. "Advances in Food Research" **7**, 171. Academic Press, Inc., New York.

Hunt, M. V., and N. A. Matheson. 1959. The relationship between the quality of dehydrated raw beef and the ATPase activity after storage at various moisture contents. *Food Research* **24**, 262.

Perry, S. V. 1953. Protein components of the isolated myofibrils. *Biochem. J.* **55**, 114.

Perry, S. V., and J. B. Chappell. 1957. The action of 2,4-dinitrophenol on myosin and mitochondrial adenosine triphosphatase systems. *Biochem. J.* **65**, 469.

Smithies, W. R., and T. S. Blakley. 1959. Design of freeze-drying equipment for the dehydration of foodstuffs. *Food Technol.* **13**, 610.

Tappel, A. L. 1956. Freeze-dried meat. II. The mechanism of oxidative deterioration of freeze-dried beef. *Food Research* **21**, 195.

Taylor, J. F., A. A. Green, and G. I. Cori. 1948. Crystalline aldolase. *J. Biol. Chem.* **173**, 591.

Some Properties of the Fibrillar Proteins of Normal and Watery Pork Muscle

J. R. BENDALL[a] AND J. WISMER-PEDERSEN
Danish Meat Research Institute, Roskilde, Denmark

(Manuscript received October 11, 1961)

SUMMARY

It is shown that the washed muscle fibrils obtained from watery pork have a lower water retention at low ionic strength, and much lower extractability at high ionic strength, than the fibrils from normal pork. These changes are accompanied by a gain of protein by the washed watery fibrils, and this protein originates from the soluble sarcoplasmic proteins. All the changes, including the characteristic gain of protein, can be artificially induced in normal meat by allowing it to pass into rigor at 37° C. The isoelectric region, or region of minimum swelling of watery fibrils, whether washed or unwashed, is similar to or slightly lower than that of normal fibrils. There is a broad isoelectric zone in both cases, extending from ~pH 5 to ~5.70. On the other hand, the IP of fully coagulated fibrils lies between 5.6 and 6.1. Washed and unwashed fibrils of watery meat show about the same degree of swelling at all pH values. Normal fibrils, however, show a higher water retention in the unwashed state than the washed. This effect is not due to the Mg or Ca ions included in the unwashed samples, but may result from interaction between the sarcoplasmic and fibrillar proteins. In the unwashed state, the swelling of normal fibrils is nearly double that of the watery fibrils at all pH values.

It is shown that the rise of pH in intact carcasses of watery meat as they cooled from 37 to 10° C was probably due to the effect of temperature on the pK of ionizable groups of the proteins and buffering substances. It can be reproduced artificially and reversibly in native and coagulated minced meat, merely by raising or lowering the temperature. The titration curves of watery fibrils show similar titration constants (pK′) to those of normal fibrils, but a loss of titratable groups. Heat coagulation, on the other hand, results not only in a bigger loss of titratable groups but in a much larger shift in the titration constants. These results can be interpreted to show that the fibrillar proteins of the watery fibrils are not denatured or aggregated in the usual sense, but are probably covered by a layer of denatured sarcoplasmic protein that is firmly bound to the surface of the myofilaments.

In recent years several reports have appeared in the literature describing in the ham and back muscles of the pig a condition that is characterized by pale color and excessive loss of fluid from the meat when the carcass is cut up for the market (Herter and Wilsdorf, 1914; Ludvigsen, 1954; Wismer-Pedersen, 1959; Briskey *et al.*, 1959; Lawrie *et al.*, 1958; Lawrie, 1960 and others). This condition was described by Ludvigsen as muscle degeneration (MD), and by Wismer-Pedersen (1959) as watery pork. Both these authors have shown that it is characterized by very rapid glycolysis post-mortem, so that the pH of the meat reaches values of <6.00 at ¾ hr after slaughter (pH_1; the pH of the longissimus dorsi muscle in the lumbar region measured by probe electrodes ¾ hr after slaughter.) Thus the meat is at a low pH and a high temperature (>35°C) during the first 1–2 hr post-mortem under commercial factory conditions. In contrast, the fall of pH in meat of normal appearance is much slower, so that the carcass has time to cool well below 25°C before the pH has fallen to 6.0. Lawrie (1960) described a similar condition in English Landrace pigs as "white-muscle disease," which is also characterized by rapid glycolysis post-mortem but is often further accentuated by excessively low ultimate pH values of <5.20, in contrast to the findings in the Danish Landrace breed, where the ultimate pH of the "watery" muscles lies in the normal range of 5.30–5.60 (Briskey and Wismer-Pedersen, 1961.

[a] Present address: Low Temperature Research Station, Cambridge.

Wismer-Pedersen (1959) and Wismer-Pedersen and Briskey (1961a) have shown that watery muscle differs in many ways from normal muscle, but particularly in the reduced extractability of the muscle proteins at high salt concentrations and in a reduced water-holding capacity. They suggested that these changes could be accounted for by denaturation and aggregation of the muscle proteins, particularly of the actomyosin filaments of the myofibrils, and this suggestion seemed to be supported by a characteristic rise in pH that occurred in the watery pork after processing was complete and while the temperature was falling to that of the cooling room.

In the present paper, we have re-examined the proteins of watery pork in rather more detail, with particular emphasis on the proteins of the muscle fibril, actin and myosin. For this purpose, we washed the fibrils free of sarcoplasmic proteins in dilute salt solutions, and then examined their water-binding capacity, protein content, and titration curves, and their extractability at high salt concentrations. We found that the washed fibrils from watery pork invariably had a higher protein content, but a lower water-binding capacity and much lower extractability than normal fibrils. On the other hand, their isoelectric point, as judged by the pH for minimal swelling, was nearly the same as or lower than that of normal meat, whereas their proton-binding capacity per g of protein was reduced. Their buffer capacity curves showed, however, maxima and minima similar to those of normal meat, and differed entirely from those of heat-coagulated fibrils, whether watery or normal.

These facts, taken together, suggest that the watery condition of pork meat is due not to aggregation or denaturation of the fibrillar proteins in the classical sense, but rather to adsorption of denatured sarcoplasmic proteins onto the surface of the fibrils and myofilaments, thus reducing the number of charged groups available for proton-binding and water-binding. This conclusion is supported by the fact that the proton-binding of normal washed fibrils is not changed by keeping them at 37°C for 1½ hours at low pH (5.40), whereas whole meat allowed to pass into rigor at 37°C shows all the characteristic symptoms of wateriness.

Against these arguments there is the finding of Briskey and Wismer-Pedersen (1961) that the pH of intact meat of the watery type apparently reaches a minimum 1½ hr after slaughter and then rises again slowly as the meat cools. pH increases of this kind could be taken to mean that considerable denaturation of the proteins had occurred (Bull, 1938; Bate-Smith and Bendall, 1946; Bendall, 1947). Examination of the pH changes in washed and unwashed fibrils, as the temperature was raised or lowered, showed, however, that the effect was entirely reversible, that is, that the pH fell as the temperature was raised and regained its original level as the temperature was once more lowered. This suggests that the effect is mainly due to changes in the pK values of charged groups on the fibrillar and sarcoplasmic proteins.

METHODS

Preparation of pork samples. To obtain meat of normal or watery type, samples of longissimus dorsi muscle were cut from the lumbar region of the carcasses 24 hr after slaughter. The pigs were of Danish Landrace breed, reared at the progeny testing station Sjælland, and slaughtered at Roskilde bacon factory after electric stunning. The pH taken ¾ hr after slaughter (pH_1) was above 6.20 in the normal muscles and below 5.60 in the watery muscles. Some samples were obtained 10 min after slaughter of the animals and were allowed to go into rigor at ~20°C. These served as controls in the titration studies.

Preparation of fibrils. Fibrils were prepared from the meat by passing it through a mincer and weighing out 5-g samples of the mince into KCl or buffer solution. The mince was then homogenized to fibril level with an Ultra-Turrax blender, which was taken to full speed in 3 bursts of 10 seconds duration, so as to avoid excessive heating. When unwashed fibrils were required, no further preparation was necessary. The preparation of washed fibrils is described later. It should be noted that homogenates prepared in this way contain pieces of connective tissue not thoroughly disintegrated by the blender. In titration studies it was necessary to cut these up finely with scissors. Microscopic examination of the homogenates revealed that all the muscle tissue proper was reduced to fibril level by the procedure.

Measurement of fibrillar volume and nitrogen content. For comparison of the fibrillar volumes

Table 1. Fibrillar protein content and water binding of normal and watery pork after washing the fibrils in 0.04*M* K phosphate (pH 7.00; I = ~0.09).

High pH_1			Low pH_1		
pH_1	Fibrillar protein (g per g meat)	g water retained per g protein	pH_1	Fibrillar protein (g per g meat)	g water retained per g protein
6.59	0.131	10.7	5.41	0.157	6.0
6.42	0.129	11.2	5.51	0.145	7.2
6.51	0.133	10.9	5.36	0.154	5.8
6.25	0.116	12.9	5.38	0.139	7.6
6.81	0.116	12.0	5.39	0.167	6.5
6.46	0.132	11.0	5.40	0.164	7.0
6.50	0.132	12.1	5.50	0.173	6.5
6.52	0.135	11.0	5.41	0.163	5.8
6.26	0.138	11.4	5.50	0.172	6.1
6.26	0.133	9.1	5.60	0.166	5.9
6.35	0.114	11.1	5.60	0.143	6.8
Mean 6.45	0.128	11.22	5.46	0.158	6.47
Ultimate pH		5.44 ± 0.036 (9)			5.39 ± 0.054 (9)
Total N % of whole meat		3.77			3.78

and nitrogen contents of watery and normal fibrils (as in Table 1), 5 g muscl ewas homogenized in 40 ml of 0.04*M* postassium phosphate (pH 7.00; ionic strength, I = 0.09) and centrifuged 10 min at 1500 × G. The supernatant was discarded and the fibrils re-mixed, with the Ultra-Turrax blender, in 40 ml of the phosphate buffer. They were then centrifuged for 10 min, the supernatant was again discarded, and the fibril layer was weighed and its nitrogen content estimated by the usual Kjeldahl procedure. The results of the nitrogen estimations were expressed as g fibrillar protein per g of meat, taking the nitrogen content of the fibrillar proteins as 16.7% (Bailey, 1937).

Extractability. To measure the extractability of the muscle proteins at high ionic strength, the minced meat was homogenized and washed as in section 3 above, except that the buffer used was 0.04*M* potassium phosphate (pH 6.50; I = 0.05). The fibril layer was then re-suspended in 40 ml of a solution of 0.5*M* KCl + 0.04*M* postassium phosphate (pH 6.5; I = 0.55), left 30 min at 0°C, and then centrifuged 30 min at 1500 × G. The supernatant fluid was decanted and the fibril residue weighed. The nitrogen content of residue and supernatant was estimated.

Preparation of samples for titration studies and measurement of isoelectric point. Five-gram samples were minced as above and homogenized in 20 ml 0.1*M* KCl. Washed fibrils were prepared from this homogenate by washing twice in 20 ml of 0.1*M* KCl. Finally the fibril layer was suspended in 20 ml of 0.1*M* KCl. Measurement of phosphate content showed that such washed fibrils contain less than 1 mol. of acid-soluble phosphate per 10^5 g of protein. For the measurement of IP or point of minimal swelling the pH was adjusted with 0.1*N* HCl or NaOH. When the IP of unwashed fibrils was to be measured, 0.004*M* iodoacetate was included in the KCl solution to prevent any possible glycolysis. In either case, the procedure was to allow the fibrils to equilibrate for 15 min at 20°C at the desired pH, and then to centrifuge them for 5 min at 1500 × G. The fibril layer was weighed after decantation of the supernatant.

Titration studies. Fibrils, prepared as above, were titrated stepwise with 0.1*N* HCl or NaOH over the range pH 1.8–11.00, care being taken to allow the samples to equilibrate for 5–10 min after each addition of acid or alkali. The pH was measured with a Radiometer pH meter, type *22*.

The titration curves are expressed as the number of protons absorbed or released per 10^5 g protein. This parameter was estimated from the equations:

$$\log \frac{C_b}{C_a} = pH_a - pH_b \qquad [1]$$

and

$$\text{mols } H^+ \text{ bound} = [V(C_b - C_a)/W] \times 10^5 \qquad [2]$$

where C_a and pH_a respectively represent the concentration of protons and the pH of a 0.1*M* KCl solution to which HCl has been added in the absence of fibrils, and C_b and pH_b are the corresponding values in the presence of fibrils; V is the volume of the solution in ml and W is the weight of protein in g. C_b can be exactly computed from pH_b from:

$$C_b = \frac{[H^+]_b}{\gamma_H} = \text{anti-log } (-pH_b - \log \gamma_H) \qquad [3]$$

where γ_H is the activity coefficient of the H^+ ion at pH_b (Cohn and Edsall, 1943). In practice the necessary correction was estimated from the pH of HCl/KCl or NaOH/KCl solutions of known concentration.

Measurement of the dependence of the pH of the meat on temperature. Washed or unwashed fibrils were prepared in 0.1*M* KCl, as in the titration studies. The pH was measured at 20°C, and then the temperature was raised slowly to 40°C (10 min) and the pH recorded as it fell back to 20°C. For measurements below 20°C the beaker containing the fibrils was cooled in an ice-water mixture to ~8°C and allowed to warm spontaneously again to 20°C. To simulate the conditions of the experiments of Briskey and Wismer-Pedersen (1961), the pH meter was set to 6.50 at 20°C, using a Sørensen buffer of that pH as standard, and the temperature adjustment of the meter was subsequently kept constant at 20°C regardless of the actual temperature. Corrections were then made by measuring the pH of the Sørensen buffer against temperature in exactly the same manner. To check the accuracy of the measurements, the variation of the pK value of 0.04*M* imidazole (pH 7.2) with temperature was measured. The heat of ionization (ΔH) derived from these data was found to be +6600 cals/mole from 8 to 40°C. The published value for the imidazole group of histidine is +6900 cals/mole (Cohn and Edsall, 1943). The agreement is seen to be satisfactory.

RESULTS

Water retention and protein content of fibrils washed at low ionic strength. Table 1 shows the water retention and protein content of fibrils of normal and watery meat prepared by washing in 0.04 phosphate buffer at pH 7.00, I = 0.09. It is seen that the fibrils from meat with low pH_1 values (<5.60) have a higher protein content (×1.23) but a lower water retention per g of protein (×0.58) than fibrils from meat with high pH_1 (>6.20). The protein content of the washed normal fibrils is a little higher than the value of 0.115 g/g muscle, given by Bendall (1961) for rabbit fibrils free of stroma. The origin of the extra protein of watery fibrils will be discussed later.

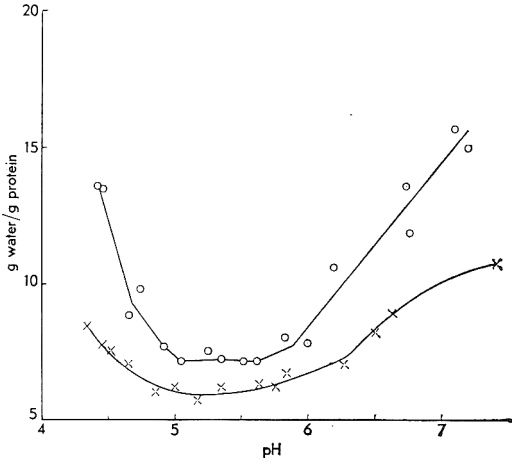


Fig. 1. Swelling curves of the washed fibrils of normal and watery pork, in 0.1*M* KCl at various pH values. Swelling expressed as g water retained per g protein, after centrifuging 5 min at 1500 x G. ⊙ samples of normal pork allowed to go into rigor at 20°C; × samples of watery pork ($pH_1 = 5.60$).

Table 2. Extraction of actomyosin at pH 6.50 (I = 0.55) from washed fibrils of normal and watery pork.

High pH_1				Low pH_1			
pH_1	(1) Protein extracted (g/g meat)	(2) Residue (g/g meat)	(1) + (2) Total fibrillar protein	pH_1	(1) Protein extracted (g/g meat)	(2) Residue (g/g meat)	(1) + (2) Total fibrillar protein
6.59	0.118	0.0146	0.133	5.41	0.0156	0.138	0.153
6.42	0.121	0.0180	0.139	5.51	0.0189	0.113	0.132
6.51	0.122	0.0156	0.138	5.36	0.0146	0.133	0.147
6.25	0.120	0.0180	0.138	5.38	0.0162	0.125	0.141
6.81	0.119	0.0118	0.131	5.39	0.0144	0.147	0.162
6.46	0.120	0.0273	0.147	5.40	0.0192	0.140	0.159
6.50	0.127	0.0135	0.140	5.50	0.0210	0.164	0.186
6.52	0.136	0.0135	0.149	5.41	0.0176	0.145	0.162
6.26	0.133	0.0125	0.146	5.50	0.0214	0.164	0.185
Mean 6.48	0.124	0.0161	0.140	5.43	0.0177	0.141	0.159
Values as % total protein	88.5	11.5			11.1	88.9	

The table also shows that the differences in water retention between normal and watery fibrils are not due to differences in ultimate pH or total nitrogen content, which are nearly identical in the two types.

Extractability at high ionic strength (I = 0.55) at pH 6.50. Table 2 shows the amount of protein extracted by a phosphate buffer of I = 0.55, pH 6.50, from fibrils washed free of soluble sarcoplasmic proteins at I = 0.05, pH 6.50. It is seen that normal fibrils (pH_i >6.20) are almost completely extracted by the procedure, giving a highly viscous solution containing 88.5% of the fibrillar proteins, whereas in the case of "watery" fibrils (pH_i <5.60) only 11% of the fibrillar proteins are extracted, and the solution is quite limpid. These extreme differences between the types are best shown up by extraction at pH 6.50, because at higher pH values the normal fibrils yield an extract so viscous that it cannot be centrifuged, and at lower pH values extraction is very incomplete. It would clearly be desirable to study in more detail the relation between extractability and pH.

The origin of the extra-fibrillar protein of watery meat. The results in Table 1 might be taken to indicate that there is a higher content of true fibrillar protein in watery meat than in normal meat. This, however, is unlikely to be the case, because an exactly similar gain in apparent "fibrillar" protein can be brought about merely by heating a minced sample of normal muscle at 37°C for 1½ hr or by allowing a sample of muscle cut from the animal immediately after death to pass into rigor at 37–41°C. In one experiment of the first type, for example, the unheated fibrils had a protein content of 0.122 g per g meat, after washing out the sarcoplasmic proteins, whereas the fibrils prepared from the heated mince had a protein content of 0.141 g per g meat. Similarly, in an experiment in which one piece of muscle was allowed to go into rigor at 20°C and the other at 40.5°C, the respective protein contents of the washed fibrils were 0.128 and 0.150 g per g meat. A similar but rather more detailed experiment is given in Table 3 to illustrate the differences in water retention, protein content, and extractability between fibrils from meat allowed to pass into rigor at 20°C and fibrils from the same meat passing into rigor at 37°C. They are again similar in magnitude to the differences between watery and normal fibrils shown in Tables 1 and 2.

Table 3. Effect of temperature of rigor on the water retention and extractability of fibrils.

Treatment	Fibrils washed at pH 7.00, I = 0.09		Fibrils extracted at pH 6.5, I = 0.55
	g H_2O retained per g protein	g fibrillar protein per g meat	(% fibrillar protein extracted)
Rigor at 20°C	11.8	0.119	81.0
Rigor at 37°C	7.7	0.137	9.4

The relation between water retention and pH in the fibrils from normal and watery pork. Fig. 1 illustrates the relation between the water retention per g of protein and the pH for washed fibrils from normal and watery pork at I = ~0.1. In this case, the normal fibrils were prepared from meat allowed to go into rigor at 20°C. It is seen that "watery" fibrils retain less water per g of protein than those of normal pork at all pH values between 4.5 and 7.5. In spite of this, the zone of minimal swelling, which is probably also the isoelectric region, is about the same in the two cases, although the zone is broader with the "watery" fibrils and extends to lower pH values. This is exactly the reverse of what happens when the proteins of normal meat are aggregated by heat, as we see from Fig. 2, which shows the water retention of fibrils

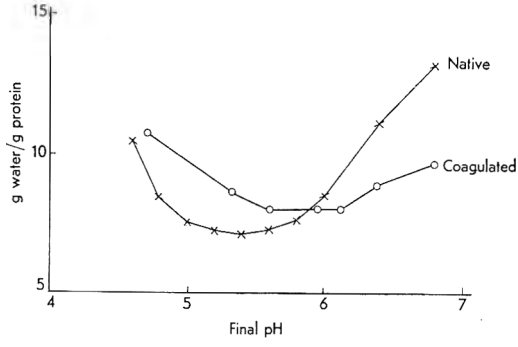


Fig. 2. Swelling curves of normal washed fibrils coagulated at various pH values at 90°C, in 0.1*M* KCl. The average curve for washed fibrils from Fig. 1 is superimposed. Results expressed as in Fig. 1.

coagulated at various pH values at 90°C. The minimum of the swelling curve is now between 5.90 and 6.00. It should be noted that the absolute values of water retention for the coagulated fibrils cannot be strictly compared with those of the native fibrils, because of the very different nature and packing density of the coagulated particles. The isoelectric point measured in this way is very close to the pH of ~6.00 attained by washed normal fibrils after coagulation under isoelectric conditions at pH 5.40. This new pH reached after coagulation must represent the isoelectric point of the coagulum, since no acid or base has been added to the system.

The similarities of the IP's of normal and watery meat can also be shown by measuring the water retention of unwashed fibrils (see Fig. 3). The curves show that the unwashed watery fibrils

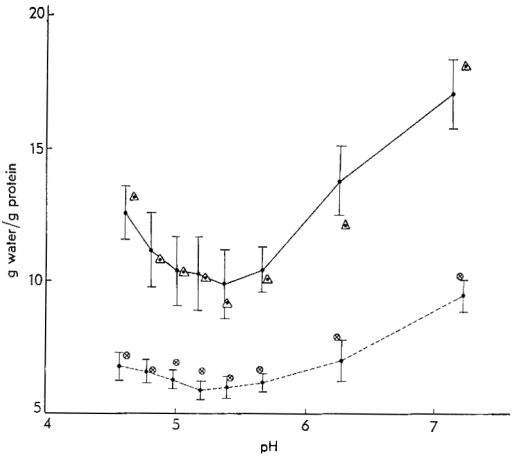


Fig. 3. Swelling curves of unwashed fibrils of normal meat (pH_i 6.30) and of watery meat (pH_i 5.60). Results expressed as g water retained per g fibrillar protein. Mean values for 3 experiments are given with the S.E.'s of the means. ——— normal; -------- watery. △ indicates an experiment with meat passing into rigor at 20°C; ⊗ indicates an experiment with the same meat passing into rigor at 37°C.

retain about the same amount of water at all pH values as the washed "watery" fibrils in Fig. 1, whereas the unwashed normal fibrils retain considerably more than the washed. Since the unwashed fibrils contain all the salts, non-protein nitrogenous compounds, and sarcoplasmic proteins of the meat, it is difficult to say which of these components is responsible for the difference in the latter case. Certainly, it is not due solely to the presence of Mg or Ca ions, since addition of these to washed fibrils in the concentrations expected to be present in the homogenates, that is, respectively ~2mM and 0.4mM, diminishes the water retention rather than increasing it. It seems more likely that the effect is due to interactions between the sarcoplasmic and fibrillar proteins, but more experiments are necessary to confirm this suggestion. It will also be noted from the figure that the isoelectric region is again somewhat lower in the watery fibrils than in the normal fibrils. Also illustrated in Fig. 3 is an experiment comparing the water retention of unwashed fibrils from meat passing into rigor at 20°C with that of fibrils from the same meat allowed to go into rigor at 37°C. It is seen that the 20°C fibrils follow the curve for normal fibrils, whereas the 37°C fibrils follow that for watery fibrils.

The reversible effect of temperature on the pH of muscle fibrils and extracts. The experiments of Briskey and Wismer-Pedersen (1961) on intact pig carcasses showed that in pigs where the pH fell rapidly (watery type 4) it also fell to the very low level of ~5.15 and subsequently rose again to ~5.50 during the cooling of the carcass. Their results are plotted as pH against temperature in Fig. 4. It will be noted that the pH tends to increase most steeply as the temperature falls from 20 to 10°C. If the rise of pH is to be taken as a measure of denaturation this feature of their curve is exactly the opposite of what would be expected, since it is well known that the rate of denaturation falls rapidly with falling temperature (Hamm and Deatherage, 1960). For this reason we have re-examined the phenomenon, using washed and unwashed fibrils, coagulated minced meat, and the Kochsaft prepared from whole meat, that is, the filtered liquid obtained after heat coagulation at 90°C. The curves in Fig. 4 show that large changes in pH occur with all the preparations studied as the temperature is raised or lowered, and that they are all inversely related to the temperature. That the effects with native fibrils are not due to denaturation is shown first by the fact that they are reversible within ±0.03 pH units at 20°C, and also that coagulated minced meat shows a comparable effect. The curves as shown have not been corrected for temperature effects on the setting of the pH meter, which was standardized to pH 6.5 at 20°C

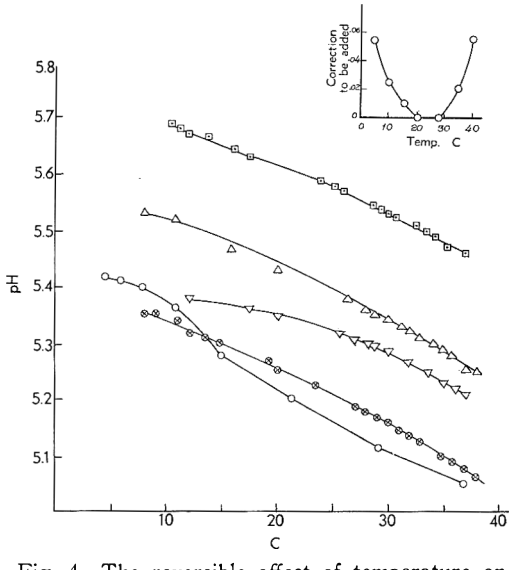


Fig. 4. The reversible effect of temperature on the pH of whole meat and various preparations of fibrils. ⊙ intact carcass (Briskey and Wismer-Pedersen, 1961); ⊗ washed fibrils (5 g meat in 10 ml 0.1M KCl); ▽ Kochsaft prepared from 10 g meat, heat-coagulated in 20 ml 0.1M KCl; △ unwashed fibrils (5 g meat in 10 ml 0.1M KCl); ⊡ coagulated mince (10 g meat + 3 ml 0.1M KCl).

Table 4. Reversible effect of temperature on pH and proton binding of various preparations of pork muscle.

Preparation	pH at			Change in H^+ bound (in 10^{-6} mols per g of meat)		
	10°C	20°C	30°C	10°C	20°C	30°C
Whole meat [Briskey and Wismer-Pedersen (1961)]	5.495	5.32	5.18	+9.75	0	−7.50
Unwashed fibrils	5.66	5.53	5.395	+7.50	0	−7.60
Coagulated mince	5.82	5.71	5.60	+6.40	0	−6.40
Washed fibrils	5.47	5.35	5.21	+3.20	0	−3.70
Expected value for sarcoplasmic proteins	.....	.....	.....	~+1.60	0	~−1.90
Kochsaft	5.51	5.45	5.35	+1.30	0	−2.10

without altering the temperature compensator from 20°C, as in the experiments of Briskey and Wismer-Pedersen. The measured corrections, shown as an inset, are seen to be quite small.

The pH/temperature curves, after correction, can be converted into numbers of protons (H^+) bound or released by the buffering substances of the meat as the temperature is changed, by employing the measured buffering capacities of each of the respective preparations. The values calculated in this way are shown in Table 4. The results of Briskey and Wismer-Pedersen have been similarly recalculated, using a buffering capacity of 54×10^{-6} moles H^+ per pH per g meat. It is seen that the unwashed fibrils show a reversible absorption or release of protons of the same order as that shown by the intact meat on the carcass.

Titration curves. To illustrate the form of the titration curves, we have chosen the curves for normal and watery fibrils in the native state (Figs. 5 and 6, respectively). The buffering capacities ($\Delta H^+/\Delta pH$) derived from these curves at intervals of 0.2–0.4 pH units are shown in Figs. 7 and 8, respectively, and also the buffering capacities of normal and watery fibrils after coagulation at ~pH 5.40 at 90°C. Since we have shown above that the washed fibrils of watery meat contain extra protein, probably in the form of denatured sarcoplasmic protein, we have also titrated the coagulated sarcoplasmic proteins of normal meat. Table 1 shows that such protein is present in watery fibrils to the extent of ~0.19 g per g of total protein, the remainder being in the form of true fibrillar protein. The calculated titration curve for the proteins in this proportion is shown in Fig. 6, and also the actual curve for 0.19×10^5 g coagulated sarcoplasmic protein alone.

We see from Figs. 5 and 6 that there are losses of titratable groups (proton binding) in both the alkaline and acid regions of the curves on passing from the normal to the watery state, although these losses do not result in any appreciable shift in the maxima and minima of the $\Delta B/\Delta pH$ curves in the acid or neutral region (Figs. 7 and 8). This is in complete contrast to the effect of coagulation, which is characterized not only by a loss of groups but also by a large shift in the maxima and minima of the $\Delta B/\Delta pH$ curves in the acid and neutral regions.

In connection with the titration curves of watery fibrils, it is of some interest that the fibrils from a sample of meat allowed to go into rigor at 40.5°C showed proton binding per g of protein almost identical to that of the samples of watery fibrils in Fig. 6, and an almost identical gain of protein of sarcoplasmic origin. By contrast, washed normal fibrils held at 40°C for 1½ hr gave a normal titration curve of the type in Fig. 5, and showed no loss of titratable groups.

DISCUSSION

The changes responsible for wateriness in pork meat have been variously ascribed to acute degeneration of the muscular tissue in the living animal (Ludvigsen, 1954), to excessively low ultimate pH values of 5.20 (Lawrie, 1960) and to a rapid fall of pH post-mortem (Briskey and Wismer-Pedersen, 1961). Although it is clear that both muscle degeneration and very low ultimate pH values might give rise to wateriness in the meat, it is most unlikely that either of these changes is responsible for the condition in the Landrace pigs studied here. In the first place, the mean ultimate pH values of the normal and the watery muscles were identical in our experiments, as they were in those of Briskey and Wismer-Pedersen (1961), and secondly we were able to confirm the observation that the watery condition could be produced merely by holding a normal piece of meat 2 hr at 33°C (Wismer-Pedersen, 1959), or by allowing rigor to occur at 37 to 41°C. Moreover, in detailed

studies of the rigor process at 37°C, to be reported elsewhere, it was observed that the muscles began to weep and their color to change only when they were about to pass into rigor, that is, when pH had fallen to 6.00 or below. It is, therefore, obvious that the immediate cause of wateriness in the Landrace pigs studied here is the combined effect of high temperature and low pH on the muscle proteins, as Wismer-Pedersen and Briskey (1961a) have shown. In fact, wateriness appears to be a general phenomenon, not confined solely to Landrace pigs, but characteristic of all muscles when rigor is allowed to take place at 37°C at low pH values, because it is then also found in a more or less severe form in the longissimus dorsi and psoas muscles of the rabbit and the ox (Bendall, unpublished observations), and to a very marked degree in whale muscle (Marsh, 1952).

Aside from their decreased extractability and water retention, watery fibrils are distinguishable from normal fibrils mainly in their higher protein content. This extra protein, derived from the sarcoplasm, is evidently present either in an aggregated form or firmly combined in some other way with the fibrillar proteins, actin and myosin. The problem is therefore to decide whether the reduced water retention and the greatly reduced extractability of watery fibrils is due simply to the deposition of this layer of sarcoplasmic protein onto the surface of the myofilaments or whether it is due to denaturation and aggregation of the fibrillar proteins themselves. Two criteria may be used to distinguish between these possibilities: first, to enquire whether a rise of pH and of isoelectric point, characteristic of the phenomenon of denaturation, can be detected as the muscles pass into the watery state (*cf.* Bull, 1938; Bate-Smith and Bendall, 1946; Bendall, 1947; Putnam, 1953); and, secondly, whether the titration curves of watery fibrils show any of the changes that characterize denatured proteins (Bendall, 1947).

As already pointed out, Briskey and Wismer-Pedersen (1961) described a rise of

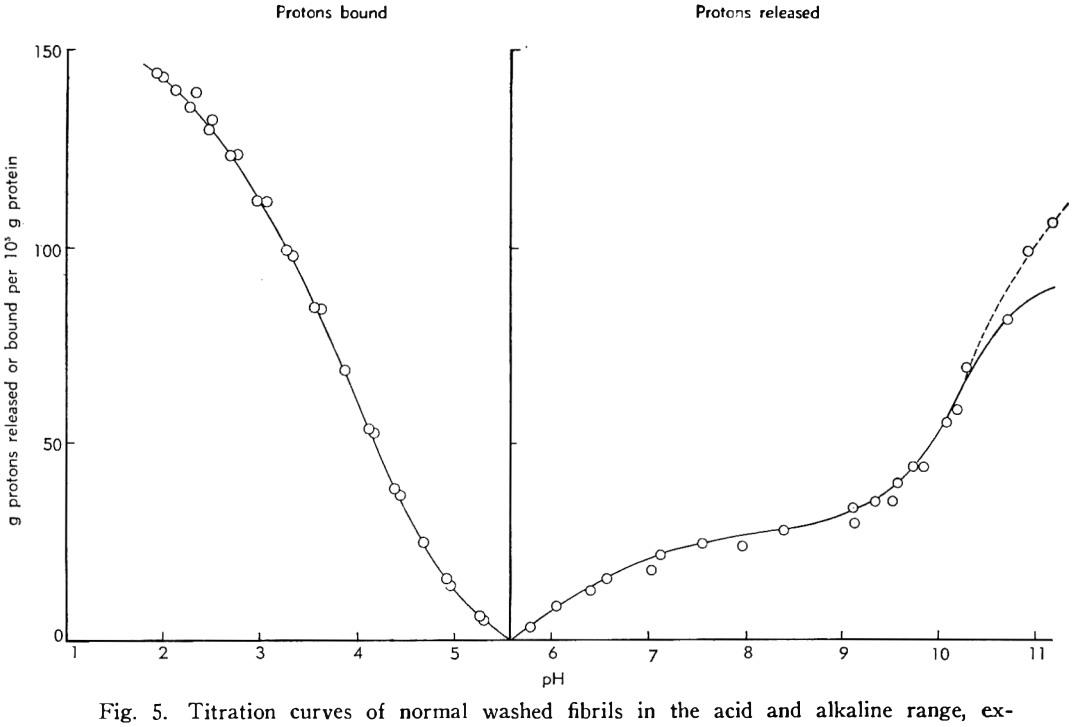


Fig. 5. Titration curves of normal washed fibrils in the acid and alkaline range, expressed as g protons released or bound per 10^5 g protein. Average fibrillar protein content = 0.128 g per g meat. Full line calculated from the constants in Table 5. ⊙ indicates the observed points.

pH in muscles of watery type, but this cannot necessarily be taken as an indication of denaturation—first, because it occurs as the temperature of the meat is falling; secondly, because it is larger than the actual rise of 0.25–0.30 units observed after the complete heat coagulation of whole meat; and thirdly, because a similar but reversible effect of temperature on pH can be demonstrated in both native fibrils and heat-coagulated whole meat (Fig. 4). The number of protons reversibly bound or released as the temperature of the unwashed native fibrils and of the heat-coagulated whole meat is raised or lowered is seen to be nearly the same in the unwashed native fibrils and in the heat-coagulated whole meat as it is in the intact carcass (Table 4). It is therefore reasonable to suppose that the effect is due to changes in the pK values of the various buffering substances of the meat with temperature.

The main groups likely to be involved in this range of pH are the imidazole and carboxyl groups of the proteins, and those of carnosine and anserine. The pK of the imidazolium groups has a negative temperature coefficient, and that of the carboxyl groups a positive one, so that in both cases protons will be released on raising the temperature, and bound again on lowering it (Cohn and Edsall, 1943). Unfortunately, we do not know the exact numbers or pK values of these groups in whole meat, so that we cannot accurately predict their effect on the proton-binding. We can, however, make the following assumptions: 1) that the 26 groups per 10^5 g of protein that titrate in the washed fibrils with a pK′ of 6.40 are imidazolium groups, and that the 105 groups titrating with pK′ = 4.15 are carboxyl groups (see Table 5); 2) that a similar number of imidazolium and carboxyl groups are present in the sarcoplasmic proteins; 3) that the total protein content is about 0.20 g per g of meat; and 4) that the heat of ionization ($\triangle$H) of the imidazolium groups is +6900 cals per mole and that of the carboxyl groups ~ -1500 cals per mole (Cohn and Edsall,

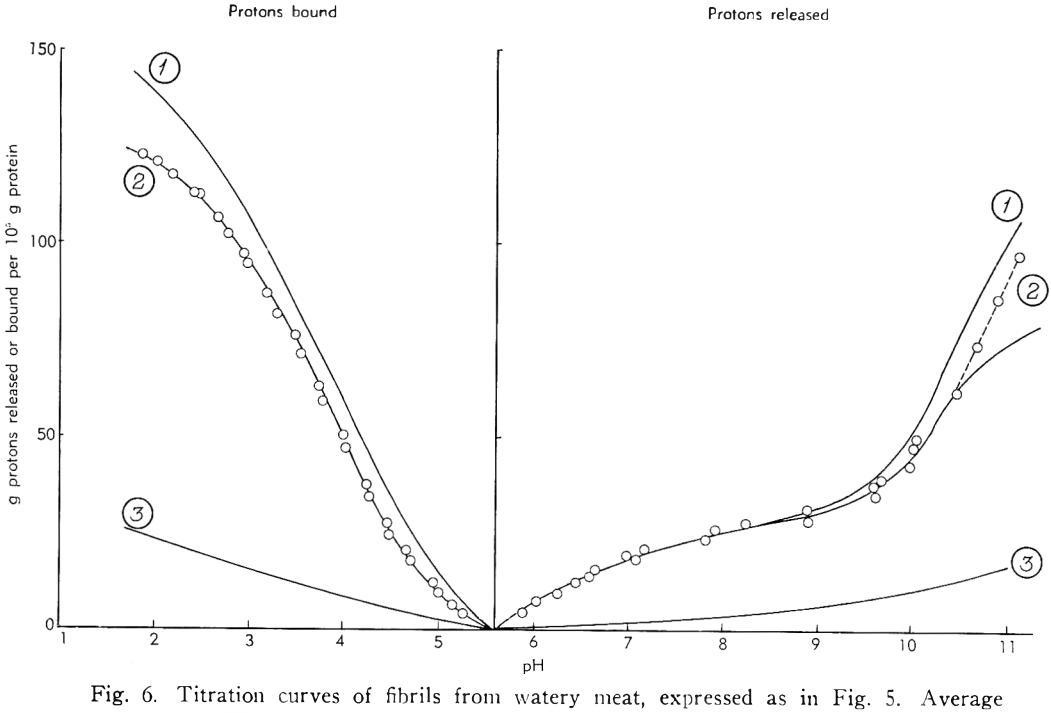


Fig. 6. Titration curves of fibrils from watery meat, expressed as in Fig. 5. Average fibrillar protein content = 0.158 g per g meat. Curve (1) is calculated from the known proton binding of 0.19×10^5 g denatured sarcoplasmic protein + 0.81×10^5 g fibrillar protein. Curve (2) is the curve calculated for watery meat from the constants in Table 5. Curve (3) is the titration curve for 0.19×10^5 g denatured sarcoplasmic protein. ⊙ indicates the observed points.

Table 5. Summary of titration constants and number of groups titrating, in various fibrillar preparations.

Type of preparation	Number of groups titrating per 10^5 g protein at pK′ =										
	2.10	2.60	3.00	3.40	3.50	4.15	4.50	4.70	6.40	6.70	10.2
Normal, native	.....	48	.....	.....	.....	105	.....	.....	26	.....	72
Normal, coagulated	.....	.....	48	.....	40	.....	.....	44	.....	26	54
Watery, native	.....	39	.....	11	.....	80	.....	.....	26	.....	57
Watery, coagulated	.....	.....	48	.....	41	.....	.....	40	.....	26	51
Sarcoplasm, coagulated	52	.....	.....	58	.....	.....	40	.....	?[a]	?[a]	?[a]
Calc. for 0.19 parts sarcoplasm to 0.81 parts fibrillar protein	10	39	.....	11	.....	85	7.5	.....	25	.....	72

[a] g-protons bound per 10^5 protein = 29 from pH 8.4 to 5.60, and 63.5 from pH 11 to 8.4.

1943). Calculating on this basis for the effect of changing the temperature of the unwashed fibrils in Table 4 from 20 to 37°C, we would expect a release of $\sim 5.2 \times 10^{-6}$ moles of H^+ against the found value of 7.3×10^{-6} moles. The discrepancy is not large when we consider that no account has been taken of the possible effect of carnosine or anserine or of the other buffering substances known to be present. We may conclude that we can account for the apparent rise of pH of watery meat in terms of the effect of temperature on the pK values more easily than by invoking a general denaturation phenomenon. Nevertheless a small rise of pH would be expected in the watery muscles as the sarcoplasmic proteins were denatured and became deposited on the fibrils. Calculating for the known amount of protein deposited in this way (Table 4), we would expect the rise of pH not to exceed 0.05 unit in the whole meat, whereas complete heat coagulation results in a rise of 0.25–0.30 unit at pH 5.40. Such a small rise would probably go undetected in the intact carcass.

The criterion of the isoelectric point (IP) similarly indicates that denaturation in the classical sense can have occurred only to a minor degree, if at all, in the watery fibrils, because their IP tends, if anything, to be lower than that of normal fibrils, whereas the IP of fully coagulated fibrils is much higher (Fig. 1–3). It is also of some interest that the swelling of unwashed normal fibrils is higher at all pH values than that of washed fibrils, whereas watery fibrils show about the same swelling in both cases. This suggests that the sarcoplasmic and fibrillar proteins are mutually active in the swelling process in normal meat but not in watery meat.

The other criterion we may apply to watery meat is that of proton-binding. For this purpose it is necessary to calculate the apparent titration constants (pK′) of each curve, which we have done by trial and error. These constants are analogous to, but not necessarily identical with, the true pK values of the groups titrating in the various regions of the curves (Cohn and Edsall, 1943). We started from the assumption that the peak in the $\Delta H^+/\Delta pH$ curve of normal meat at pH 4.15 (Fig. 7) represented the pK′ of a class of similar groups, virtually undisturbed by the presence of other groups in the region pH 3.65–4.65. We then applied the general equations:

$$pH = pK'_q + \frac{\log a_q}{1 - a_q} \qquad [4]$$

$$n\,a_q = n\left\{\frac{\text{antilog}\,(pH - pK'_q)}{1 + \text{antilog}\,(pH - pK'_q)}\right\} \qquad [5]$$

where pK′ is the titration constant of the qth class of groups; a_q is the fraction of the total number of groups in the class that have given up protons to base at any pH; and na_q represents the number of protons given up by groups of the qth class at any pH. It follows from these equations that 52% of the groups in the class with pK′ = 4.15 should titrate between pH 3.65 and 4.65. We actually find 54.5 g protons given up per 10^5 g protein over this range of

pH, so that the total number of groups with pK 4.15 is ~105. Removing these groups from the observed curve, as they would titrate according to equation 5, we found by similar reasoning that there was a class of 48 groups with a pK′ of 2.60 in the acid region, and a class of ~26 groups with a pK of 6.40 in the neutral region. In the alkaline region, there appeared to be a class of 72 groups with pK 10.20, and this would mean that the remaining 50 or 60 basic groups expected to be present from the analytical data of Kominz *et al.* (1954) should have pK′ values greater than 11.00 (Mihalyi, 1950). Unfortunately, this could not be checked with the electrode assembly available, which had a large "alkaline" error above pH 11.40.

From these calculations it is possible to describe the titration of the native fibrils of normal meat from pH 1.8 to 10.2 as follows:

g protons bound =

$$48a_1 + 105a_2 + 26a_3 + 72a_4 \qquad [6]$$

$$= 48\left\{\frac{\text{antilog (pH–2.6)}}{1 + \text{antilog (pH–2.6)}}\right\}$$

$$+ 105\left\{\frac{\text{antilog (pH–4.15)}}{1 + \text{antilog (pH–4.15)}}\right\}$$

$$+ 26\left\{\frac{\text{antilog (pH–6.4)}}{1 + \text{antilog (pH–6.4)}}\right\}$$

$$+ 72\left\{\frac{\text{antilog (pH–10.2)}}{1 + \text{antilog (pH–10.2)}}\right\}$$

The curve calculated from this equation (Fig. 5) is seen to agree well with the observed values for two different fibril preparations up to the limit at pH 10.2.

A similar type of analysis was applied to the curves for watery fibrils and for the coagulated fibrils of normal and watery meat. The results of the analyses (Table 5) are in terms of the classes of pK′ values and the number of groups titrating in each class. To demonstrate the general validity of these constants we have plotted the average values of the observed results against the values calculated from Table 5 for the particular complex case of coagulated normal fibrils (Fig. 9). It is seen that the agreement is good in the acid range, but small discrepancies occur in the alkaline range after about 30 groups have been titrated (pH 8.2–10.0), and a larger discrepancy after 60 groups have been titrated (pH 10.4 and above). The first discrepancy may be due to the constant at pH 6.70 being too low, or to the release of α-amino and -groups, not taken into account in the calculations. The second discrepancy arises because we do not know the pK′ values of the groups alkaline to pH 11.0. As a further check, we may note that the calculated curve for watery fibrils (curve 2, Fig. 6) also agrees well with the observed points, up to the limit at pH 10.2. From these arguments we may have reasonable confidence in the constants (pK′) given in Table 5.

Since it is believed that this study of the titration constants of native, watery, and coagulated fibrils is the first of its kind, it is necessary to enquire whether the data for the native fibrils agree with the amino acid analysis and titration curves of the main fibril proteins, actin and myosin. Mihalyi (1950), for instance, showed that purified myosin contained 165 titratable acid groups, 16 imidazolium groups, and 134 basic groups per 10^5 g of protein. There are no comparable data for actin, but the amino acid analysis of Kominz *et al.* (1954) shows the presence of 117 free acid groups, 19 imidazolium groups, and 109 free basic groups. If the fibrillar proteins contain 1 part of actin to 2 parts of myosin (*cf.* Hanson and Huxley, 1957), there should therefore be ~148 acid groups and ~18 imidazolium groups per 10^5 g. We find a total of ~153 acid groups and ~26 groups titrating with a pK′ of 6.40, which are likely to be imidazolium groups (Cohn and Edsall, 1943). Similarly we find 72 groups titrating with a pK′ of 10.20, close to the expected pK values of tyrosine and lysine (Cohn and Edsall, 1943). The analytical data of Kominz *et al.* (1954) would yield 23 tyrosine and 74 lysine groups for an actin-myosin ratio of 0.5. The over-all agreement is, therefore, fairly good, particularly since we have not taken any account of

the stroma, connective tissue, and tropomyosin undoubtedly present in our preparations. These components, however, probably cancel each other out, because connective tissue has a small number of charged groups and tropomyosin a very large number. According to Lawrie (1960) and Hanson and Huxley (1957), they are present in the fibrillar proteins in about equal amounts of 10%. In this connection, we may note that Wismer-Pedersen and Briskey (1961b) found normal whole meat to bind 144.4 g equivs of safranin per 10^5 g total protein, whereas watery meat bound 187 g equivs per 10^5 g. This measure of the number of acidic groups in normal meat agrees well with the present results, which would show about 146 g protons to be bound at pH 1.8. In watery meat, however, the proton binding is not likely to exceed 130 g protons per 10^5 g total protein. It is difficult to account for the large discrepancy in the latter case, although it may be pointed out that it is not certain that the dye-binding method is an exact measure of proton binding, particularly where protein-protein interactions may have taken place in the material under study. For example, Hamm and Deatherage (1960), using this method, detected a loss of acidic groups after heat denaturation of whole beef muscle, but could find no comparable loss of basic groups, which would have been expected from the results of the present titration studies.

Inspection of Table 5 shows that the proton binding of the washed watery fibrils is lower than normal in both the acid and alkaline regions, but that this decrease is not accompanied by the characteristic shifts in the titration constants observed after complete heat coagulation. On the other hand, the titration constants of watery fibrils are shifted by heat coagulation in much the same manner as those of normal fibrils, particularly in the acid and neutral regions. This again suggests that most of the protein of watery fibrils is not in a denatured or coagulated form. If this is so, however, we must seek some other explanation of the decreased water retention and extractability of watery meat. A possible explanation can be suggested from the titration studies. We have shown that the extra protein of watery fibrils must arise from the sarcoplasm, and yet when we calculate the expected contribution of this protein to the total proton binding we find that nearly all of it has disappeared from the actual titration curves of the watery fibrils (Table 5). The only way in which this could happen would be for the missing groups to have become linked to groups on the fibrillar proteins. We may

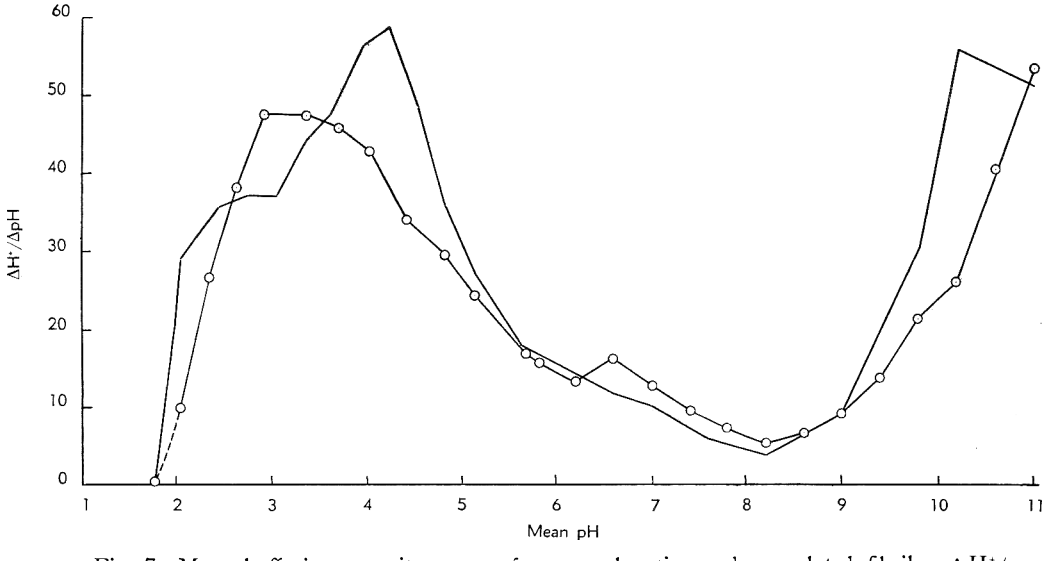


Fig. 7. Mean buffering capacity curves for normal native and coagulated fibrils. $\Delta H^+/\Delta pH$ = g protons bound per pH unit per 10^5 g protein. Plain line = native fibrils; ⊙ = coagulated fibrils.

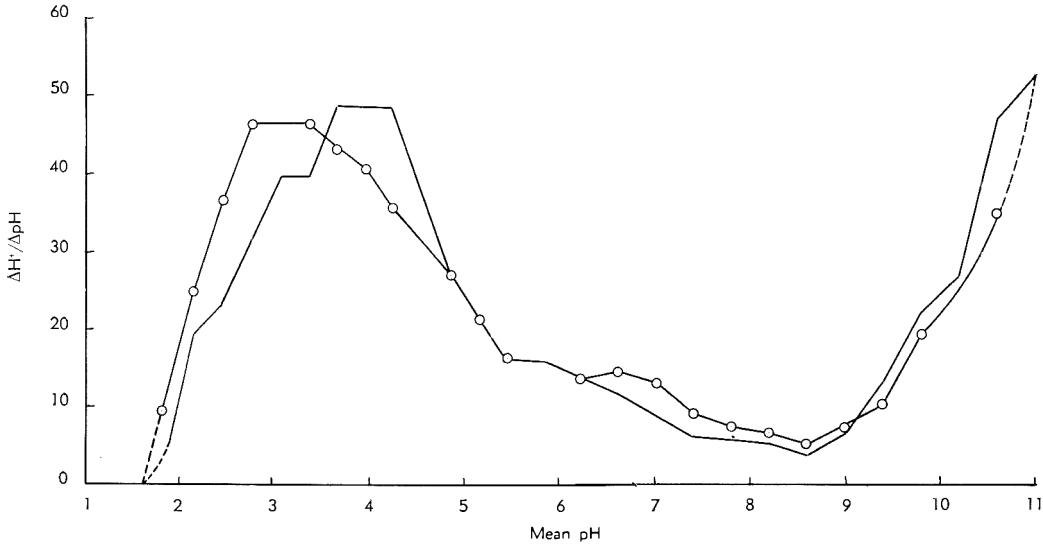


Fig. 8. Mean buffering capacity curves for the native and coagulated fibrils of watery pork, expressed as in Fig. 7.

assume, for instance, that the carboxyl groups titrating with pK′ values of 2.60 and 4.15 belong to the fibrillar proteins, whereas the groups with pK′ 3.40 belong to denatured sarcoplasmic proteins. This would mean that all the 11 groups with pK′ 2.10 and the 7.5 groups with pK′ 4.50 contributed by the sarcoplasmic proteins have become linked to the fibrillar proteins. This could occur by the formation of bonds between these groups and the hydroxyl groups of tyrosine or even the ϵ-amino groups of lysine, or the guanidino groups of arginine of the fibrillar proteins. In addition, 5 carboxyl groups with pK 4.15 have been lost from the fibrillar contribution, possibly by linkage to basic groups on the sarcoplasmic proteins. The over-all effect should be a loss of 23 or 24 groups titrating in the alkaline range. We actually observe a loss of ~15 groups with pK′ 10.20, and it is possible that the remainder are lost at more alkaline pH values. These losses are, indeed, similar in magnitude to those observed after the complete coagulation of normal fibrils, which in the acid region amount to 25 groups and in the alkaline region to ~18 groups, although in the latter case the missing carboxyl groups must have become linked to hydroxyl or basic groups, either on the same molecule or between molecules of the same protein type, which might account for the greater shift in the pK′ values of neighboring groups. Similarly, complete heat coagulation of watery fibrils must result in the breaking of some of the postulated bonds between the fibrillar proteins and the layer of denatured sarcoplasmic proteins on their surface, because we

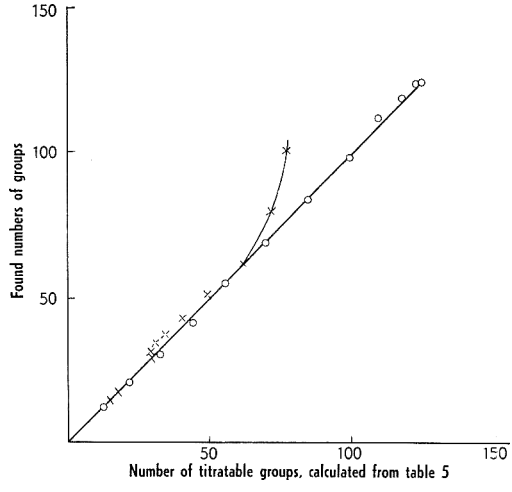


Fig. 9. Plot of calculated numbers of titratable groups in normal coagulated fibrils against the average number actually found at any given pH value (see Table 5). ⊙ values from pH 5.60 to 1.80, taking pH 5.60 as the starting point; × values from pH 5.60 to 11.20, taking pH 5.60 as the starting point.

find after coagulation considerable shifts in pK values that are similar in kind, but not in degree, to those observed in coagulated normal fibrils.

We may conclude that we have an example in watery meat of a unique type of protein-protein interaction, where the main fibrillar protein, actomyosin, is in the native form but has become covered with a layer of denatured sarcoplasmic protein that is bound to it sufficiently strongly to make it resistant to extraction at high, and to hydration at low, ionic strengths. This change in the structure of the muscle, unlike the effect of coagulation, leaves the titration constants of the combined proteins more or less unaltered, with the result that the isoelectric point of watery fibrils is the same or even slightly lower than that of normal fibrils.

ACKNOWLEDGMENT

We thank Miss L. Jensen and Miss B. Mogensen for laboratory assistance in this work.

REEFRENCES

Bailey, K. 1937. Composition of the myosins and myogens of skeletal muscle. *Biochem. J.* **31**, 1406.

Bate-Smith, E. C., and J. R. Bendall. 1946. Heat coagulation of muscle proteins. *Nature* **156**, 632.

Bendall, J. R. 1947. The effect of heat-denaturation on the base-binding capacity of beef-muscle press-juice. *Proc. Roy. Soc. London B.* **134**, 272.

Bendall, J. R. 1961. A study of the kinetics of the myofibrillar adenosine triphosphatase of rabbit skeletal muscle. *Biochem. J.* **81**, 520.

Briskey, E. J., and J. Wismer-Pedersen. 1961. Biochemistry of pork muscle structure. I. Rate of anaerobic glycolysis and temperature change versus the apparent structure of muscle tissue. *Food Sci.* **26**, 297.

Briskey, E. J., R. W. Bray, W. G. Hoekstra, P. H. Phillips, and R. H. Grummer. 1959. The chemical and physical characteristics of various pork ham muscle classes. *J. Animal Sci.* **18**, 146.

Bull, H. B. 1938. Protein denaturation. *Cold Spring Harbor Symposia Quant. Biol.* **6**, 140.

Cohn, E. J., and J. T. Edsall. 1943. "Proteins, Amino-Acids and Peptides." pp. 89, 449, 454. Reinhold Publishing Corp., New York.

Hamm, R., and F. E. Deatherage. 1960. Changes in hydration, solubility and charges of muscle proteins during heating of meat. *Food Research* **25**, 587.

Hanson, J., and H. E. Huxley. 1957. Quantitative studies on the structure of cross-striated myofibrils. II. Investigations by biochemical techniques. *Biochim. et Biphys. Acta* **23**, 250.

Herter, M., and G. Wilsdorf. 1914. Die Bedeutung des Schweines für die Fleischversorgung. Deut. Landwirtschafts-Gesellschaft. Berlin, Heft, 270.

Kominz, D. R., A. Hough, P. Symonds, and K. Laki. 1954. The amino-acid composition of actin, myosin, tropomyosin and the meromyosins. *Arch. Biophys. Biochem.* **50**, 148.

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

Lawrie, R. A., D. P. Gatherum, and H. P. Hale. (1958). Abnormally low ultimate pH in pig muscle. *Nature* **182**, 807.

Ludvigsen, J. 1954. Undersøgelser over den såkaldte "muskel-degeneration" hos svin. Beretning fra forsøgslaboratoriet, København, I. 272.

Marsh, B. B. 1952. Observations on rigor mortis in whale muscle. *Biochim. et Biophys. Acta* **9**, 127.

Mihalyi, E. 1950. Binding of ions by myosin. II. The effect of neutral salts on acid-base equilibria of myosin. *Arch. Biochem. Biophys.* **41**, 107.

Putnam, F. W. 1953. In "The Proteins." Vol. 1, Part B, pp. 850–3. Neurath, H., and Bailey, K., eds. Academic Press, Inc., New York.

Wismer-Pedersen, J. 1959. Quality of pork in relation to rate of pH change post-mortem. *Food Research* **24**, 711.

Wismer-Pedersen, J., and E. J. Briskey. 1961a. A biochemistry of pork muscle structure. III. On the relationship of post-mortem acidity and temperature. *Food Technol.* **15**, 232.

Wismer-Pedersen, J., and E. J. Briskey. 1961b. Rate of anaerobic glycolysis versus structure in pork muscle. *Nature* **189**, 318.

APPENDIX

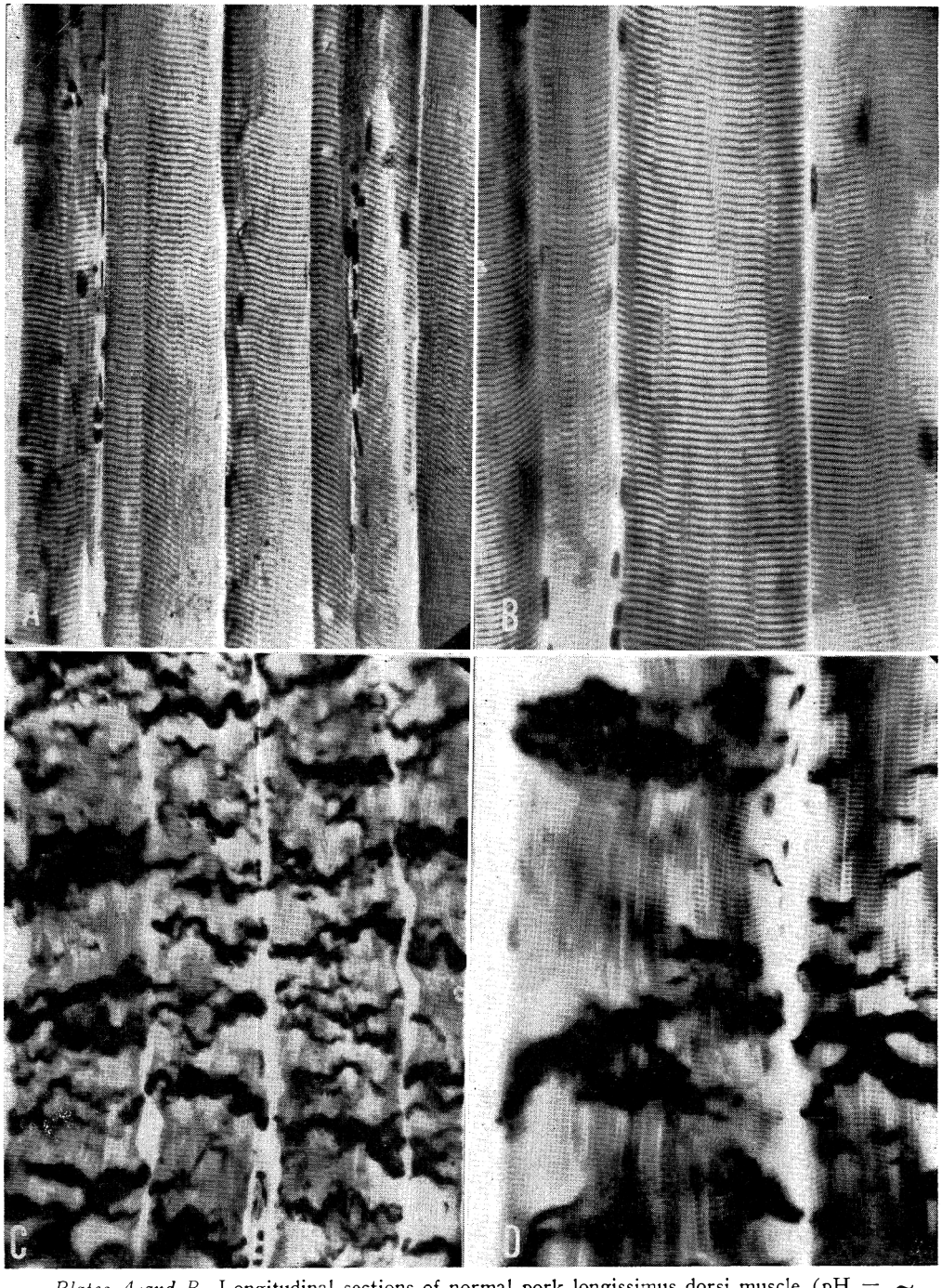
In collaboration with N. R. King, we obtained samples of normal and watery pork muscle from Danish Landrace pigs for histological examination in Cambridge. The accompanying plates illustrate the results.

The samples of meat, excised from the lumbar region of the longissimus dorsi muscle, were fixed in 10% neutralized formol saline and embedded in celloidin after dehydration. Longitudinal sections were then cut, stained with Heiderhain iron hemotoxylin and differentiated in iron alum.

Plates A and C show longitudinal sections of normal and watery muscle at a magnification of ~300×. It is seen that the characteristic cross-striations are uniform and clear in the normal section, whereas in the section of watery muscle, many heavily-staining dark bands of protein lie irregularly across the fibers. The minimum width of these bands is at least 6× that of the cross-striations themselves, that is, at least 20 mμ. They are, however, entirely irregular in pattern. As we see from the more highly magnified section (600×) in plate D, the bands of protein seem to cover one muscle fiber in each case, and in some places small bulges appear in the sarcolemma at the points where the bands terminate. It will also be noted that the cross-striations can still be distinguished and are only slightly distorted, where they underlie a dark band. It can be concluded, therefore, that the basic muscle structure is still intact in the watery meat, so that there is no evidence from these sections, or from many others like them, that any degeneration had occurred in the muscle during the life of the animal, as might perhaps have been supposed from the name *muskel-degeneration,* given to the symptoms by Ludvigsen.

Although there is no positive way of proving the point from histology alone, it is nevertheless likely that the irregular dark bands are composed of denatured sarcoplasmic proteins, and that they are, in fact, the origin of the extra "fibrillar" protein of watery meat, which has been shown in the previous paper to render the fibrils resistant to extraction at ionic strengths normally sufficient to extract actomyosin. Similar bands were reported by Lawrie *et al.* (1958) in meat with very low ultimate pH. He interpreted these results to mean that the fibers had become internally disrupted, which would indeed be the case if denaturation of the sarcoplasm had occurred. Unlike some of Lawrie's sections, the present samples do not show any kinking or waving of the fibers; on the contrary, they appear to run flat in the plane of the section and in the plane perpendicular to it.

We may conclude that the main change in structure that characterizes watery pork is the deposition of denatured protein of sarcoplasmic origin, in the form of dark-staining bands lying transversely within the muscle-fibers. From the evidence of the previous paper, this change occurs only after the death of the animal—while its rigor processes are reaching completion—and can be attributed to an unusually rapid production of lactic acid, leading in its turn to a low pH value while the meat is still at a high temperature, that is, to conditions specially conducive to denaturation.



Plates A and B. Longitudinal sections of normal pork longissimus dorsi muscle (pH = ~ 6.50), magnified, respectively, 300× and 600×.

Plates C and D. Longitudinal sections of watery pork (pH = ~ 5.50), magnified respectively, 300× and 600×.

The Effect of Sudden Cooling on the Respiration of Pea Tissue

DOHN G. GLITZ,[a] PAUL A. BUCK,[b] ANDREW C. RICE,[c] AND WILLIAM D. POWRIE[d]

(Manuscript received April 24, 1961)

Thermal shock may be defined as an injury to biological materials that occurs when the materials are cooled rapidly to a temperature near their freezing point without the actual formation of ice crystals in the tissue. A distinction should be made between the terms "thermal shock" (or "temperature shock"), which results from the cooling of the tissue, and "physiological cold injury," which may occur when tissues are exposed to low temperatures above freezing for comparatively long periods. Both of these phenomena have been considered in the review by Smith (1954), although the same terminology has not been employed.

Thermal shock has been noted upon the cooling of a number of materials including sperm (Birillo and Puhaljski, 1936), bacteria (Mayer, 1955), and human red blood cells (Lovelock, 1954), but no report was found that describes the phenomenon in a tissue of a higher form of plant such as might be used for food. On the other hand, physiological cold injury has been observed in a number of foods (Smith 1954), where it occurs as an injury during cold storage. No experiments have attempted to demonstrate alterations in respiratory activity that could be interpreted as thermal shock, using a common food tissue, green peas, which is not known to be sensitive to this type of injury.

Each instance of thermal shock previously cited used a different measure of the degree of injury, since in each case the injury took a different external form. Studies of sperm have usually been based on measurement of motility of fertility (Smith, 1954), while lysis was measured in the case of red blood cells (Lovelock, 1954) and viability in the case of bacteria (Sherman and Cameron, 1934). While recent results indicating a cold lability of certain enzymes (Pullman *et al.*, 1960; Raijman and Grisolia, 1961) cannot be considered as evidence of thermal shock at this level of organization, they do encourage possible detection of thermal shock using a metabolic measurement. Certain tissues and cells, such as spermatozoa, liver, kidney, intestines, skin, and blood (Mann and Lutwak-Mann, 1955; Mayer, 1955; Sherman, 1959; Smith *et al.*, 1956), as well as bacteria (Mayer, 1955; Sherman, 1959) and enzymes (Hultin, 1955), have been studied for thermal shock with metabolic techniques, the success varying with the material studied.

In a study of physiological cold injury in cucumbers, Eaks and Morris (1956) found respiratory changes to be associated with the injury, and Appleman and Smith (1936) reported alterations in respiratory activity as a result of prolonged cold storage of a number of vegetables.

On the basis of this literature it was decided to examine respiratory activity in pea tissues in the hope of detecting a metabolic alteration that could be interpreted as thermal shock.

Sherman (1959) wrote: "Although the goal in food preservation is not viability but palatability of foods after storage, the approach in construing methods of preservation of life by freezing could profitably be applied to food. The following are some of the considerations in such an approach: (a) Some plant and animal cells are sensitive to the phenomenon of temperature shock, manifested in the deleterious effects of rapid cooling above the freezing temperature. It is necessary to ascertain whether

[a] Research Assistant, University of Wisconsin, Madison, Wisconsin. Present address: University of California, Berkeley, California.

[b] Cornell University, Ithaca, New York.

[c] Seneca Grape Juice Corporation, Dundee, New York.

[d] University of Wisconsin, Madison, Wisconsin.

or not the material to be frozen is sensitive and if so to attempt to obviate this by slower cooling or with protective substances. It would be futile to attempt preservation by freezing if cells were injured even before freezing temperatures were reached. (b) The rate of cooling during ice formation is also a vital factor. Slower rates have been more beneficial to some cells and tissues, faster rates to others. Each type of material should be evaluated as to its optimal rate rather than accepting that found successful with other material. The possible beneficial role of protective substances should also be explored. (c) Once desirable rates of cooling above and below the freezing temperature are found experimentally, the final temperature reached is examined as to effect, since the tolerated minimum temperature may also vary from substance to substance. The factor of time at this temperature naturally follows in consideration of effects during storage. (d) Finally, the rate of rewarming from the frozen state during dissolution of ice has often proved critical. Techniques employed in the approach may include macroscopic and microscopic observations on structural alterations, chemical and biochemical analysis, termograph recording of temperature changes, various physiological and cytochemical tests of viability and injury, and freeze-drying for study of ice formation, among others.

MATERIALS AND METHODS

Experimental materials. The test material was green peas *(Pisum sativum)* of the variety Perfected Wales, generously supplied by Dr. D. Hagedorn, of the Department of Plant Pathology of the University of Wisconsin. No sudden or appreciable changes in temperature were allowed to occur during their growth in a greenhouse. The peas were harvested at a stage of maturity at which they were judged suitable for use as food.

Preparation of samples. The peas were prepared for measurement of respiratory activity by slicing them into sections about 1/16 in. thick. This was done with a series of double-edged razor blades spaced equally with metal washers and held together as a unit by bolts through the centers of the washers and blades. Thirty to sixty grams of peas were prepared for each experiment, and the slices mixed to promote uniformity. Samples were about 1 g each, weighed to the nearest 0.005 g on an analytical balance. Variations in weight were corrected in the calculations.

Pretreatment of peas. In some experiments, peas were pretreated in an attempt to sensitize them to thermal shock, using a method similar to that of Lovelock (1954). Five ml of 1.0*M* sodium chloride solution was added to the sample in the reaction vessel, and the peas were allowed to soak 30 min at room temperature. The sodium chloride solution was then decanted, the peas were rinsed with 2 ml of distilled water, and further treatments were applied as in all other cases.

Cooling of peas. A number of methods were used to cool the peas to a temperature near 0°C in an attempt to induce a measurable respiratory change. When no pretreatment was used, the peas were chilled by placing them in a cooler kept at about 0°C immediately after the peas were sliced. The temperature at the center of the slice was measured with imbedded thermocouples. After reaching a temperature of 2–3°C (less than 5 min required), the slices were immediately returned to room temperature for weighing and measurement of respiratory activity. Multiple cooling treatments were applied by repetition of the cooling cycle.

In some cases peas were cooled in their pods by placing them in the cooler for about 10 min. They were then returned to room temperature for slicing, weighing, and measurement of respiratory activity. When desired, the cooling process was repeated before removal of the shell.

In one experiment, shelled and unshelled peas were exposed to about 0° for periods up to 24 and 48 hours, respectively. Samples were withdrawn at various times and sliced and weighed for measurement of respiratory activity.

When pretreatment was used the peas were cooled in their reaction vessels after slicing and weighing. The vessels containing the samples were placed in an ice-water bath to cool and returned to room temperature by placing them in a water bath at 25°. Multiple cooling treatments were applied by repetition of the cycle.

Measurement of respiratory activity. The evolution of carbon dioxide and the uptake of oxygen were measured in the Warburg respirometer (Umbreit *et al.*, 1957). Vessels of about 15 ml capacity were used, and the temperature was maintained thermostatically at 25° in all experiments where temperature was not the variable. Each flask contained 2 ml of distilled water in addition to 1 g of experimental material. Buffer was found to be unnecessary since pH remained nearly constant at 6.2–6.3. The evolution of carbon dioxide in an atmosphere of nitrogen was deter-

Table 1. Summary of respiratory measurements on replicate samples of pea tissue.

Sample no.	Oxygen consumed (μl/g-hr)	Carbon dioxide evolved (μl/g-hr)	Carbon dioxide evolved in nitrogen (μl/g-hr)	Respiratory quotient[a]
1	240	232	150	1.04
2	204	267	161	1.20
3	233	239	139	1.07
4	242	248		1.11
5	197	244		1.09
Median	233	244	150	1.09
Av.	223	246	150	1.10
Std. deviation	18.8	11.8	9.0	0.054

[a] Respiratory quotient values calculated using average oxygen uptake value and individual carbon dioxide evolution values.

mined after flushing the vessels with nitrogen for 15 min.

The effect of temperature on the respiratory activity of pea slices was measured. The temperatures, 0, 5, 10, 15, 20, 25, 30, 35, and 50°, were maintained thermostatically. All calculations are expressed as gas volume exchanged at standard temperature and pressure per gram of experimental material.

RESULTS AND DISCUSSION

Replication of data. A typical set of observations is presented in Table 1, showing ordinary variation between samples. From these and many similar results, average variations of less than 10% of the control value were considered to be of doubtful significance. The data presented in all other tables and in the figures represent the average of 2–6 determinations.

Respiratory activity of peas cooled after shelling. Respiratory activities were measured in uncooled peas and in peas cooled one, two, and three times to temperatures near 0°. The results are summarized in Table 2.

Comparison of the data reveals no indication of an alteration in respiratory rates, the observed differences being accounted for by experimental variation. Repetition of the cooling treatment did not produce an additive effect, as would be expected in the occurrence of thermal shock.

Respiratory activity of peas cooled before shelling. The respiratory activities of peas cooled in their shells and of uncooled peas were measured. The data (Table 3) indicate no respiratory changes indicative of thermal shock.

Respiratory activity of pretreated peas. Respiratory activity was measured in pea slices pretreated in an attempt to sensitize them to thermal shock. Table 4 shows the respiratory activity of uncooled peas and of peas cooled one, two, and three times.

The observed differences in respiratory rates can again be accounted for by normal experimental variation. Repetition of the cooling process results in an apparent reaction opposite to that which seems to result from a single cooling. This result is in contrast to that observed in the hemolysis of red blood cells (Hultin, 1955).

Respiratory activity of stored peas. Shelled and unshelled peas were held at

Table 2. Respiratory activity of peas cooled after shelling.

Sample	Oxygen consumed (μl/g-hr)	Carbon dioxide evolved (μl/g-hr)	Carbon dioxide evolved in N_2 (μl/g-hr)	Respiratory quotient
Control	226	318	190	1.40
Cooled once	224	329	178	1.46
Cooled twice	248	366	193	1.47
Cooled three times	225	321	214	1.43

Table 3. Respiratory activity of peas cooled in the shells.

Sample	Oxygen consumed (μl/g·hr)	Carbon dioxide evolved (μl/g·hr)	Carbon dioxide evolved in N_2 (μl/g·hr)	Respiratory quotient
Control	245	333	186	1.37
Cooled	226	296	180	1.32

0 and 25° for periods up to 48 and 24 hours, respectively. Samples were withdrawn at intervals, and the respiratory activity measured. The data are illustrated in Fig. 1.

No alteration in respiratory activity indicative of thermal shock or physiological cold injury is apparent in these results. With all samples, a significant drop in respiratory activity occurs within the first 1.5 hr, and then the curves begin to diverge. The only effect then apparent is that the rate of change in respiratory activity is slower in peas stored at 0° than in peas stored at 25°. The slight increase (following the initial drop) in respiratory activity of peas stored at the lower temperature is in agreement with the results of Appleman and Smith (1936).

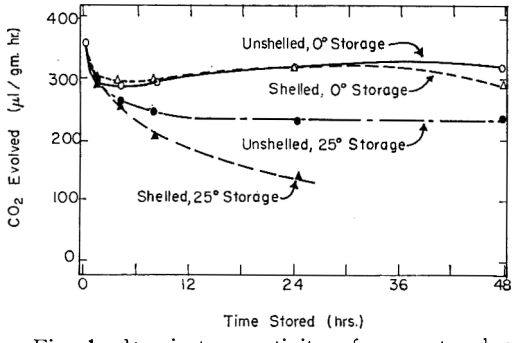


Fig. 1. Respiratory activity of peas stored at 25 and 0°C.

Effect of temperature on respiratory activity. The effect of temperature on the respiratory activity of pea slices was measured. The evolution of carbon dioxide in both air and nitrogen is seen in Fig. 2 to give a linear plot of log activity vs. 1/T (°K), corresponding in each case to an activation energy of about 12,300 calories per mole, or a Q_{10} of two. Such a linear response would be expected of a reaction controlled by one enzyme or enzymes with similar energies of activation.

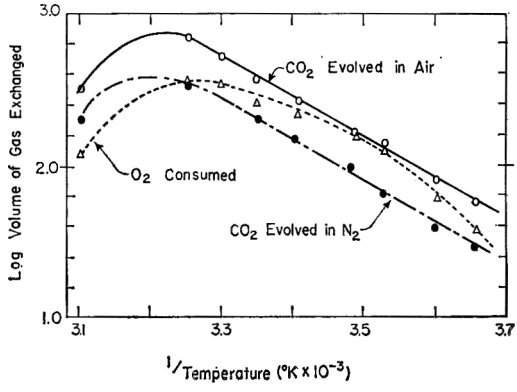


Fig. 2. Effect of temperature on the respiratory activity of pea slices.

The uptake of oxygen, on the other hand, does not give a linear plot. This may be taken as an indication that the process is not limited by enzymic action, or that more than one enzyme with different energies of activation, is involved. Separate identical experiments with a second variety of peas, Charter, gave the same result.

Fig. 3 shows the variation in respiratory quotient of RQ (volume of CO_2 evolved/volume of O_2 consumed) with temperature. It can be seen that great variations in the respiratory activity occur as the temperature is altered. This variation cannot be taken

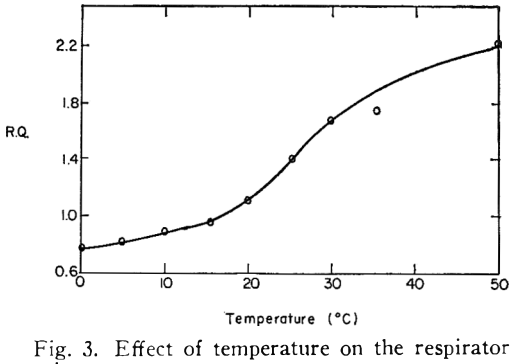


Fig. 3. Effect of temperature on the respiratory quotient.

Table 4. Respiratory activity of pretreated peas.

Sample	Oxygen consumed (μl/g-hr)	Carbon dioxide evolved (μl/g-hr)	Respiratory quotient
Control	150	275	1.82
Cooled once	148	250	1.68
Cooled three times	165	285	1.72

as an indication of thermal shock, however, since the alteration in respiratory activity could not be demonstrated to be permanent. The method of detecting thermal shock by respiratory activity might not be suited to pea tissue, although it is suitable for certain tissues, yet macroscopic and microscopic observations also failed to indicate thermal shock.

CONCLUSIONS

None of the experiments indicated thermal shock or short-term physiological cold injury of pea tissue. No permanent alterations in respiratory activity could be attributed to cooling. Macroscopic and microscopic observations also failed to indicate thermal shock, so that this phenomenon probably does not occur in pea tissue.

REFERENCES

Appleman, C. O., and C. L. Smith. 1936. Effect of previous cold storage on the respiration of vegetables at higher temperatures. *J. Agr. Research* **53**, 557.

Birillo, I. M., and L. H. Puhaljski. 1936. Problems of prolonged storage of bull and ram semen. *Problemy Zhivotn.* **10**, 24. Animal Breeders Abstr. **5**, 219.

Eaks, I. L., and L. L. Morris. 1956. Respiration of cucumber fruits associated with physiological injury at chilling temperatures. *Plant Physiol.* **31**, 308.

Hultin, E. 1955a. The influence of temperature on the rate of enzymic process. *Acta Chem. Scand.* **9**, 1700.

Hultin, E. 1955b. Calculations on the influence of temperature on the rate of biological processes. *Pubbl. staz. zool. Napoli* **27**, 300.

Lovelock, J. E. 1954. Physical instability and thermal shock in red cells. *Nature* **173**, 659.

Mann, T., and C. Lutwak-Mann. 1955. Biochemical changes underlying the phenomenon of cold shock in spermatozoa. *Arch. Sci. Biol.* **39**, 578.

Mayer, D. T. 1955. Ph.D. thesis. Published partly as *Missouri Agri. Expt. J. Ser.* 1539.

Pullman, M. E., H. S. Penefsky, A. Datta, and E. Racker. 1960. Partial resolution of the enzymes catalyzing oxidative phosphorylation. *J. Biol. Chem.* **235**, 3322.

Raijman, L., and S. Grisolia. 1961. New aspects of acetylglutamate action. Cold inactivation of frog carbamyl aspartate synthetase. *Biochem. Biophys. Research Communs.* **4**, 462.

Sherman, J. K. 1959. Preservation by refrigeration and freezing. Chapt. 6, "Manual of Food Processing," Cornell University (mimeo).

Sherman, J. M., and G. M. Cameron. 1934. Lethal environmental factors within the natural range of growth. *J. Bacteriol.* **27**, 341.

Smith, A. U. 1954. Effects of low temperatures on living cells and tissues. In "Biological Applications of Freezing and Drying." (R. Harris, ed.) Academic Press, New York.

Smith, J. T., D. T. Moyer, and C. P. Merilan. 1956. Effect of egg yolk and its isolated constituents upon the dehydrogenase activity of bovine spermatozoa. *J. Dairy Sci.* **39**, 552.

Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1957. "Manometric Techniques." 3d ed. Burgess Publishing Company, Minneapolis, Minnesota.

Studies on the Volatile Components of Different Varieties of Cocoa Beans

S. D. BAILEY, D. G. MITCHELL,[a] M. L. BAZINET, AND C. WEURMAN[b]
Pioneering Research Division, Quartermaster Research and Engineering Center
Natick, Massachusetts

(Manuscript received August 13, 1961)

SUMMARY

Volatile compounds in the aroma of five varieties of roasted and unroasted (raw) cocoa beans have been identified by mass spectral analysis and gas chromatography. The five common varieties selected for this study all contain the following compounds usually in this order of abundance: isovaleraldehyde, isobutyraldehyde, propionaldehyde, methyl alcohol, acetaldehyde, methyl acetate, *n*-butyraldehyde, and diacetyl. An additional eight compounds appear in smaller amounts. As evidenced by gas chromatographic analysis, the raw bean aroma contains the same components but in lower concentrations. The principal differences between varieties are shown to be due to the ratios of these compounds rather than new compounds. The effect of roasting period on the concentration of four aldehydes in the aroma of the ground bean is shown.

Roelofsen (1958), in reviewing information on the fermentation, drying, and storage of cocoa beans, wrote that the "chocolate flavor and odor developed on roasting is by far the most important characteristic of cocoa beans." A search of the literature has revealed that the volatile components of the roasted cocoa bean have not been studied extensively.

Of the compounds previously identified in cocoa, few may be considered to be sufficiently volatile to be associated with the aroma characteristics of the roasted bean. α-Linalool (Bainbridge and Davies, 1912), the principal component of the essential oil in Ecuadorian cocoa bean (Arriba), is a C_{10} carbon ester. Theobromine, 3,7-dimethyl xanthrene, with an m.p. of 337°C, which changes very little during the roasting process (Chatt, 1953), was once believed to develop an aroma suggestive of chocolate, but this view is no longer held (Forsyth and Rombonts, 1956 a, b). At least nine polyphenols were separated and identified by Forsyth (1957). These large molecules have long been supposed to be the precursors of aroma in tea, coffee, and cocoa. Organic acids known to occur in cocoa bean are well recognized in labeling beans according to acidity, astringency, and bitterness. Extraction of polyphenols and organic acids from the unroasted bean prevented development of the flavor on roasting (Roelofsen, 1958). Replacement of the extracted compounds did not restore the characteristic aroma. However, had these components been themselves carried through the roasting step, aroma enhancement might have occurred. The fermented and unfermented beans were shown by Maly (1955) to contain the amino acids aspartic and glutamic acids, alanine, cysteine, leucine, serine, threomine, methionine, and arginine.

Those volatile components in cocoa which have been studied include diacetyl and acetylmethyl carbinol reported by Schmalfuss and Barthmeyer (1932) and Schmalfuss and Rethorn (1935). The presence of methyl, ethyl, isobutyl, isoamyl, and dimethyl amine

[a] Present address: Walter Baker Chocolate Co., General Foods Corp., Dorchester, Mass. Work initiated during active duty training under U. S. Army Reserve Program.

[b] Present address: Central Institute for Nutrition and Food Research, T.N.O., Utrecht, The Netherlands. NAS-NRC Visiting Scientist at the QM R&E Center from December 1959–April 1961.

have been observed in cocoa powder by Weurman and De Rooy (1961). More recently, Mohr (1958) studied the composition of chocolate aroma and published initial results on the gas chromatography of chocolate conching. Mohr observed 23 compounds by gas chromatography in the solvent extract of the conched bean, but none were identified. The low order of concentration of the aroma components did not permit use of a direct sampling technique. In the present study, direct aroma sampling of the freshly ground bean was used extensively, thus permitting an examination of the cocoa bean aroma in a manner completely analogous to perception by the nose, i.e., there is no loss of components or change in ratios between components taking place in this direct sampling technique, as may occur when concentration is required. This type of gas chromatographic analysis has become possible through new, very sensitive detectors, and in this study the β-ray ionization type was used. For purposes of identification, analysis by mass spectrometry was used in accordance with procedures used in the study of volatile components of food (Merritt *et al.*, 1959).

EXPERIMENTAL

Sample. In order to study a practical method for the evaluation of aroma composition, some representative types of cocoa beans (*Theobroma cacao* L.) were selected that are well recognized in the chocolate industry for their particular characteristics. The following types of cocoa beans were used: *Accra*, a basic strong chocolate flavor, lacking the undesirable astringency and bitterness found in poorer-flavor varieties; *Arriba*, recognized by its unique and very perfumy aromatic character, though it generally has a moderate astringency and bitterness; *Bahia*, a relatively acid type of flavor, which has a basic chocolate taste but little or no aromatic quality; *Sanchez*, generally a harsh, spicy type of flavor that is masked by strong astringency and bitterness unless the beans are well-fermented; *Trinidad*, with a strong basic chocolate character similar to that found in Accra, and a typical "winey" type of aromatic flavor that is not found in other varieties.

The above are average characterizations. Actually, ranges of variation are wide from crop to crop and from bag to bag within a single shipment. This is caused by the difficulties in keeping the processing of the beans in the field under close control. Fermentation followed by air drying to a 5–7% moisture content is accomplished in the area in which the bean is grown.

The roasting process, which brings out the characteristic flavor of the cocoa bean, takes place in the chocolate manufacturer's plant. The final moisture content is of the order of 1.5–2.5%. The sample was roasted by placing the whole bean in a circulating hot air oven at 300°F. Unless otherwise indicated, the roasting period was 30 min.

Because of known differences within a given variety of bean due to curing operations, several samples were taken from each of six different commercial lots of the Accra variety. A comparison of the aroma composition of these samples showed only minor differences, which were in part due to limitations of the analytical method. For the other varieties two commercial lots from which several samples were selected were considered adequate.

Thirty grams of the roasted or unroasted (raw) bean were ground in a small laboratory blender and immediately placed in a 250-ml Erlenmeyer flask covered with a rubber serum cap. After 2 hr at room temperature to reach equilibrium, samples ranging from 1 to 5 ml of headspace over the samples were removed by glass syringe and introduced to the gas chromatography instrument. Initially, freezing and grinding with dry ice were used to prevent possible loss of heat-labile compounds or alteration in the ratio of volatile compounds. It was later found that this was not required, and, further, that excessive carbon dioxide in the headspace reduced the amount of cocoa bean volatiles in the sample taken for analysis. In the experiments below, only the first-mentioned method of preparing the samples was used.

Gas chromatographic analysis. Vapor samples from the headspace over the ground material were introduced into a Pye-Argon gas chromatograph equipped with a 20-mc strontium-90 ionization detector.

The 120-cm × 4-mm column was packed with 10% di-*n*-decyl-phthalate on firebrick. A flow of 95 ml/min of argon at a column temperature of 50°C was the standard experimental condition employed. Different column materials, at lower and higher temperatures and different flow rates, had been explored, and the above conditions selected for optimum results. A temperature programmed run from approx 5 to 75°C provided a greater separation of components, but with no increase in the number of peaks over those observed under isothermal conditions. When information on the identity had become available from mass spectrometric analysis, small quantities of the pure reference compound were added to the vapor of the cocoa bean samples to locate the

position of the compound on the chromatograph. The increase, without distortion, of the unknown peak by the known compound provided further confirmation. The latter method was found to be of considerable value because of its very great sensitivity.

Mass spectral analysis. The mass spectral technique employed has been described (Bazinet and Merritt, 1959). Approx 1×10^{-5} moles of the vapor was transferred to a sampling bottle by high-vacuum low-temperature condensation. The gaseous sample was then fractionated directly into a Consolidated Engineering Model 21-103C Analytical Mass Spectrometer at low temperature and high vacuum. Separated components were identified by comparison of the fragmentation pattern with those of known compounds.

RESULTS

Fig. 1 shows a gas chromatogram of the volatile components released from a typical sample of roasted ground cocoa bean. Resolved peaks, identified by the methods described, are correspondingly labeled. Other varieties, as is subsequently shown, give quite similar gas chromatographic patterns. Table 1 shows the mass spectral analysis giving the mole percent computed on a water- and carbon-dioxide-free basis for a typical roasted sample. The base line of the chromatogram of Fig. 1 indicates compounds identified from mass spectral evidence but present in quantities too small for resolution by gas chromatography.

The several samples from a given lot and several lots of a given variety gave very reproducible

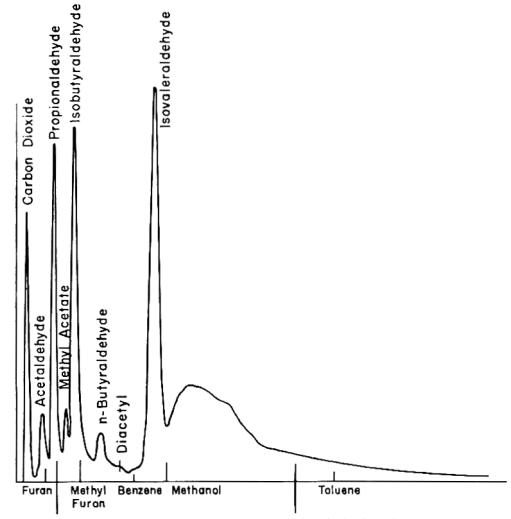


Fig. 1. Gas chromatogram of the volatile components of roasted ground cocoa. Typical chromatogram with peaks identified from mass spectral data and pure reference compounds.

Table 1. Mass spectral analysis of roasted Bahia cocoa bean vapor.

	Mole %
Isovaleraldehyde	42.0
Isobutyraldehyde	15.4
Propionaldehyde	13.0
Methyl alcohol	9.1
Acetaldehyde	7.0
Methyl acetate	6.3
n-Butyraldehyde	3.0
Diacetyl	2.8
Dimethyl sulfide	0.3
Dimethyl disulfide	0.2
Ethyl alcohol	0.2
Furan	0.2
Toluene	0.1
Benzene	0.1
Methyl furan	Trace
Acetone	Trace

results. Slight changes in the ratios of certain components were observed in some cases during reproducibility studies. Since the beans are an agricultural product it is not unusual to find such variation as a result of soil conditions, maturity differences, and variations in processing.

It was also considered to be of some interest to determine the effect of storage on a single lot of roasted cocoa beans. A sample held 4 months at room temperature was compared to an identical sample held 4 months at −15°C. The resulting gas chromatograms showed no significant differences.

In the chromatograms of the roasted samples, isovaleraldehyde (3-methyl butanal) is the most pronounced component in the varieties studied. The next major component is isobutyraldehyde, with propionaldehyde and acetaldehyde completing the four major aldehydes in the vapors of the roasted bean.

Five varieties of cocoa bean, raw, are compared in Figs. 2, 3, and 4. The importance of roasting

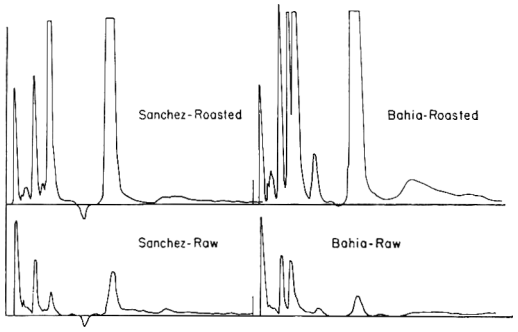


Fig. 2. Gas chromatograms of the volatile components of ground Sanchez and Bahia varieties, roasted and raw.

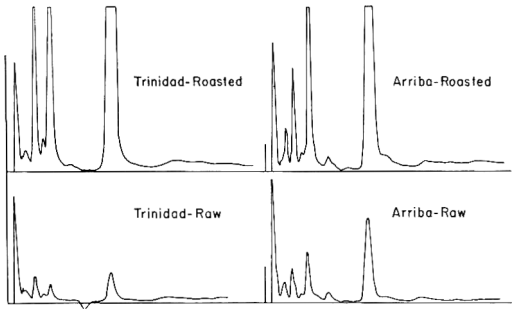


Fig. 3. Gas chromatogram of volatile components of ground Trinidad and Arriba cocoa beans, roasted and raw.

to enhancement of volatile components is observed in all varieties. A few of the minor components are not detectable in the raw bean.

The chromatograms of the unroasted Sanchez, Trinidad, Arriba, and Accra samples are very similar with respect to the relative amounts of three major components and a number of minor components. The Bahia variety, in both the raw and roasted samples, has a much higher relative amount of methyl acetate than do the other four varieties. These same five varieties were directly compared by measuring the peak areas of the four major aldehydes in chromatograms in which sample and sensitivity of the instrument permitted all peaks to be retained on the chart paper. The Accra roasted sample gave the largest total area for these four compounds, with the Bahia next and the Arriba the least. The Arriba cocoa bean is generally considered as having a high aromatic character, but this does not appear to be associated with the volatile components compared in this study.

Fig. 5 shows the effect of roasting period on the release of aldehydes. The peak heights of isovaleraldehyde, isobutyraldehyde, propionaldehyde, and acetaldehyde for roasting periods of 20, 30, 40, and 50 min are compared to another sample of the same lot that was not roasted. The release of higher aldehydes is markedly increased by 20 min of roasting. The propionaldehyde and acetaldehyde are not significantly affected by the roasting process. Roasting times of 30 and 40 min gave no significant increase over the 20-min roasting. Increased roasting time of 50 min, however, does appear to increase the release of the two higher aldehydes. From a flavor standpoint there was a preference by tasters for the 40- and 50-min samples, with five preferring the 40-min sample and three preferring the 50-min samples. One taster indicated no preference.

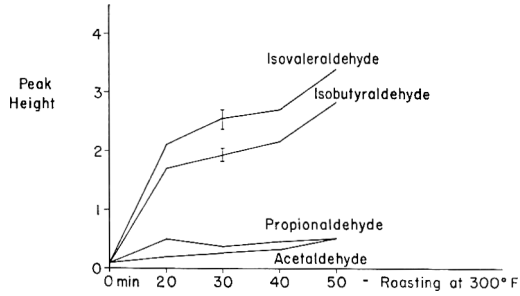


Fig. 5. Change of gas chromatogram peak heights of four volatile components with roasting period.

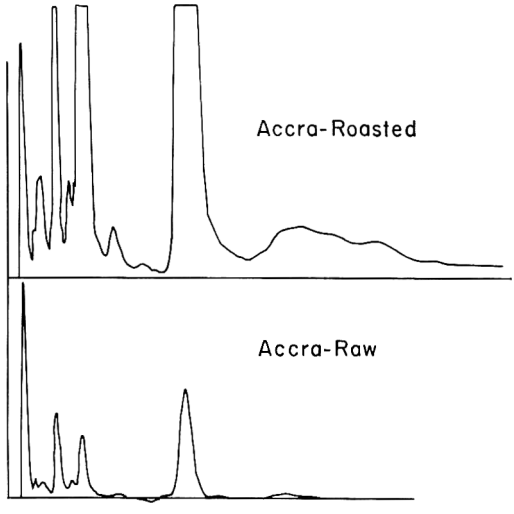


Fig. 4. Gas chromatograms of the volatile components of ground Accra cocoa bean, roasted and raw.

To obtain a general impression of the actual amounts of material represented by the chromatographic peaks, the amount of isovaleraldehyde in the chromatograms shown has been estimated by taking aliquot vapor samples from a 5.0-L spherical glass vessel in which μl amounts of the aldehyde were introduced. The quantity of aldehyde used permitted complete vaporization. It was therefore possible by taking out small gaseous samples to estimate rather accurately the quantity of isovaleraldehyde associated with a given peak height. At a detector voltage of 1750 V, a sensitivity of 1/3 maximum, and a flow rate of 95 ml/min on 10% di-*n*-decyl-phthalate, peaks covering the range of a 24-cm recording chart paper correspond to amounts up to 1.3 μg or 1.5×10^{-8} moles. In the chromatograms of Figs. 2, 3, and 4 the quantity of isovaleraldehyde is, therefore, of the order of 2 to 3×10^{-8} moles.

The threshold perception of isovaleraldehyde was determined by sniffing dilute aqueous solutions of the aldehyde. Molal concentrations of 9.3×10^{-3}, 4.6×10^{-4}, and 2.3×10^{-5} respectively gave strong, faint, and very faint but detectable odor of the aldehyde. The same size of Erlenmeyer flask was used in this odor threshold study as was used in the sampling of ground cocoa beans; in this way, the headspace sampling may be considered analogous. This has given some indication of the levels of odor threshold perception, and the amount of isovaleraldehyde associated with the peak heights observed in the cocoa samples.

It then was considered desirable to see if the compounds responsible for the chromatogram, as shown in Fig. 1, could be related to the fresh aroma of the ground roasted cocoa bean. From previous chromatographic runs, the time required for elution at room temperature was determined precisely. A 20-ml headspace sample was then introduced, with the column disconnected to prevent overloading of the detector and to facilitate trapping. The volatile compounds were trapped at dry-ice temperature in a sand-filled spiral glass trap. Upon completion of trapping the trap was allowed to come to room temperature and the contents gently flushed out. The aroma emerging from the trap was found to be characteristic of the cocoa bean and essentially the same as the headspace sample introduced. The compounds identified, therefore, appear to be of importance in characterization of the roasted cocoa bean aroma.

To what extent these volatile components may continue to be observed in the next step of the chocolate manufacturing process, was determined by gas chromatographic analysis of a sample of chocolate liquor. This sample, prepared from Bahia roasted cocoa beans, was the same sample given the 30-min roast in the roasting-period study. The gas chromatogram of the headspace sample is shown in Fig. 6. Peak A does not appear in the roasted or raw samples. Peak B corresponds to acetaldehyde, C to propionaldehyde, D to methyl acetate, E to isobutyraldehyde; F and G are new; and H corresponds to isovaleraldehyde. The conditions for the analysis represented in this chromatogram correspond to conditions for the analysis resulting in the chromatograms of Figs. 1, 2, 3, and 4. The quantity of the volatile components is therefore markedly reduced in the formation of the chocolate liquor. A more detailed study is necessary to determine the aroma composition of the liquor. The chromatogram shown for the liquor would indicate the usefulness of this technique for such additional studies.

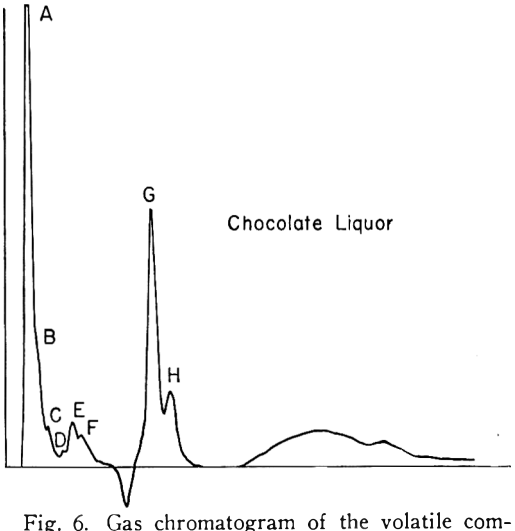


Fig. 6. Gas chromatogram of the volatile components of chocolate liquor.

DISCUSSION AND CONCLUSIONS

The aroma composition of these five varieties of roasted and raw cocoa bean was found to be very similar. The major differences were observed in the amounts of each component as shown by the gas chromatograms. A much larger concentration of methyl acetate was found in the Bahia variety, and *n*-butyraldehyde was present in the Bahia and Arriba bean to a greater extent than in other varieties.

It is generally recognized that fermentation produces a rapid breakdown of protein to produce soluble nitrogen such as amino acids (Becker and Stelling, 1952). The amino acids thus formed, through processes of oxidation, deamination, and decarboxylation during fermentation and on drying, may well serve as precursors to the aldehydes reported in this study. The general reaction for these processes, first established by Strecker in 1861, is given as follows:

$$\mathrm{RCH(NH_2)-COOH} \xrightarrow{(O)} \mathrm{R-C(=NH)-COOH} \xrightarrow[(-NH_3)]{H_2O}$$

$$\mathrm{R-C(=O)-COOH} \xrightarrow{(-CO_2)} \mathrm{RC(=O)-H}$$

The occurrence of isovaleraldehyde in cocoa bean, as reported in this study, may come from leucine through these reactions. Jack-

son and Morgan (1954) attributed the malty aroma in dairy products to the conversion of leucine to isovaleraldehyde by *Streptococcus lactis* var. *maltigenes*. By similar reasoning the presence of isobutyraldehyde and other aldehydes reported here may be attributed to similar reactions. Further studies are indicated to substantiate this observation.

Roasting at 300°F increases the amount of isovaleraldehyde and isobutyraldehyde, the most pronounced effect occurring after 20 min at this temperature. Roasting periods of 30, 40, and 50 min gave somewhat smaller increases. In this connection the prolonged heating at the elevated temperature might be expected to result in the loss of the more volatile components. The failure of acetaldehyde and, to a lesser extent, propionaldehyde to show increased amounts on roasting may be accounted for in this way.

It remains as a future problem to attempt reconstitution of a cocoa aroma from these components as well as to fortify substandard beans with some of these to determine if acceptable aroma enhancement can be achieved.

While some evidence has been presented for the importance of these compounds in chocolate liquor, a greater attention must be given to this problem. The heating and grinding in the process of preparing the liquor would be expected to result in the loss of the more volatile components.

Finally it may be concluded that analytical techniques are now available for the study of fresh and processed foods in small quantities without the requirement for extraction and concentration of volatile components. This permits an assessment of aroma composition to a degree of reliability heretofore not possible.

REFERENCES

Bainbridge, J. S., and S. H. Davies. 1912. The essential oil of cacao. *J. Chem. Soc. (London)* **101**, 2209.

Bazinet, M. L., and C. Merritt. 1959. The determination of volatile components of foodstuffs. II. Direct fractionation of a sample on the mass spectrometer and the use of low ionization voltage spectra. *QM R&E Research Rept.* (Anal. Chem. Series No. 11) June.

Becker, E., and O. Stelling. 1952. Über das Eiweiss der Kakaobohne. *Zucher-u. Süswarenwirtch* **5**, 936.

Chatt, E. M. 1953. "Cocoa." Interscience, N. Y.

Forsyth, W. G. C. 1952a. Cacao polyphenolic substances (fractionation of the fresh bean). *Biochem. J.* **51**, 511.

Forsyth, W. G. C. 1952b. Cacao polyphenolic substances (changes during fermentation). *Biochem. J.* **51**, 516.

Forsyth, W. G. C. 1955. Cacao polyphenolic substances (separation and estimation on paper chromatograms). *Biochem. J.* **60**, 108.

Forsyth, W. G. C. 1957. Cacao polyphenolic substances (the anthocyanin pigments). *Biochem. J.* **65**, 177.

Forsyth, W. G. C., and J. E. Rombonts. 1956a. Private communication to P. A. Roelofsen.

Forsyth, W. G. C., and J. E. Rombonts. 1956b. Curing of cacao beans. *Advances in Food Chem.* Vol. 8, p. 280.

Jackson, H. W., and M. E. Morgan. 1954. Identity and origin of the malty-aroma substances from milk cultures of Streptococcus lactis var. maltigenes. *J. Dairy Sci.* **37**, 1316.

Maly, A. 1955. Prumysl potravin 6, 133–7; Cacao-bean proteins. *Chem. Abstr.* **49**, 8522i.

Merritt, C. Jr., S. R. Bresnick, M. L. Bazinet, J. T. Walsh, and Pio Angeglini. 1959. Determination of volatile components of foodstuffs. Techniques and their application to studies of irradiated beef. *J. Agr. Food Chem.* **7**, 784.

Mohr, W. 1958. Untersuchungen über das Kakao-Aroma unter besonderer Berüchsichtigung des Conchierens von Schokoladenmassen. *Fette Seifen Anstrichmettel* **60**, 661.

Roelofsen, P. A. 1958. Fermentation, drying and storage of cacao beans. "Advances in Food Chemistry," Vol. 8. Academic Press. pp. 225–296.

Schmalfuss, H., and H. Barthmeyer. 1932. Nachweis von Diacetyl und Methylacetyl-carbinol in Lebensmitteln. *Z. Untersuch. Lebensm.* 63, 283.

Schmalfuss, H., and H. Rethorn. 1935. Bestimmung des Diacetyls. *Z. Untersuch. Lebensm.* **70**, 233.

Weurman, C., and C. De Rooy. 1961. Volatile amines in the odors of food. *J. Food Sci.* **26**, 239.

The Carotenoids of Meyer Lemons

A. LAURENCE CURL
Western Regional Research Laboratory,[a] Albany, California

(Manuscript received September 28, 1961)

SUMMARY

The carotenoids have been examined in the peel and pulp (the edible portion) of Meyer lemons. The principal carotenoid in the pulp was cryptoxanthin. The peel carotenoids included a number of unusual substances, including cryptoxanthin mono- and diepoxides and fractions tentatively identified as hydroxy derivatives of phytoene, phytofluene, and zeta-carotene. An unusual polyene was also found. Apparently it contains two allylic hydroxyl groups, one of which is allylic to the conjugated double bond system.

The carotenoids of Valencia oranges, both peel and pulp (the edible portion), have been investigated in considerable detail (Curl, 1953; Curl and Bailey, 1954, 1955, 1956). The carotenoids of Ruby Red grapefruit (Curl and Bailey, 1957a), tangerines (Curl and Bailey, 1957b), and navel oranges (Curl and Bailey, 1961) have also been reported. The present paper reports a study of the carotenoids of Meyer lemons. The peel was found to be a rather good source of a number of unusual carotenoids, including cryptoxanthin mono- and diepoxides and substances tentatively identified as monohydroxy derivatives of phytoene, phytofluene, and zeta-carotene.

EXPERIMENTAL

The Meyer lemons used were grown in El Cerrito, California. The fruit selected had orange-yellow peels. The pulp and peel were extracted separately: 1000 g of pulp and three lots of peel of 500, 1800, and 1100 g.

The pulp in 200-g batches was blended with 300 ml of water containing 12 g of magnesium carbonate. Filter aid (Celite 545; no endorsement implied in product identifications), 10% of the weight of the pulp, was added, and the mixture filtered on a Buchner funnel precoated with filter aid. The filter cake was worked up as previously described by Curl and Bailey (1959a), with one modification. After evaporation of the acetone from the acetone extract, the residue was transferred to a separatory funnel with water and ether, and *ca.* 2 g of sodium chloride and methanol (*ca.* 10% of the volume of the aqueous solution) were added. Addition of methanol results in cleaner and much more rapid separations in the ether extraction. The extract was saponified (Curl and Bailey 1959a) in a one-phase system of ether and 20% potassium hydroxide in methanol.

The peels, in 100-g batches, were blended with 400 ml of water containing about 2 g of magnesium carbonate. Filter aid (about 20% by weight of the peels) was added, and the mixture worked up in the same manner as the pulp.

Both saponified extracts were subjected to 100-transfer countercurrent distribution runs in a Craig apparatus with solvent system I (hexane-99% methanol, 1.8 to 1 by volume), in order to separate the carotenoids into (a) hydrocarbons, (b) monols, and (c) diols and polyols (Curl, 1953). The distribution curve for peel carotenoids indicated a more complex mixture, so a second run was made, with 200 transfers, using system I.

The diol-polyol fractions obtained with system I were further fractionated by a second 100-transfer countercurrent distribution run with solvent system IVa (hexane–75% methanol, 1:1 v/v); the latter system is similar to the previously described system IV (hexane–73½% methanol, Curl, 1960b). The contents of each tube were diluted with acetone to 50 ml, and the color measured in an Evelyn photoelectric colorimeter using filter 440.

The fractions were chromatographed on columns of magnesia (Westvaco Sea Sorb 43, 12 by *ca.* 80 mm without a diluent), using the same series of graded eluants described by Curl and Bailey

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

(1959b) for use with magnesia 2642 (diluted with an equal volume of filter aid). Spectrophotometric data were obtained with a Cary recording spectrophotometer model 14.

RESULTS AND DISCUSSION

The carotenoid contents of the pulp and peel were found to be 2.4 and 5.6 mg per kg (as beta-carotene). These values are much lower than those obtained with Valencia oranges—24 and 98 for pulp and peel, respectively (Curl and Bailey, 1956).

Countercurrent distribution. The fractional compositions of the carotenoid mixtures of the peel and pulp are given in Table 1, as well as the N_{100} values (tube number of maximum per 100 transfers) obtained with systems I and IVa. In both peel and pulp there was an unusually high percentage of monols (including epoxides), higher than in any fruit previously reported. In other fruits, such as Valencia orange peel (Curl and Bailey, 1956), Ruby Red grapefruit peel (Curl and Bailey, 1957a), tangerine peel (Curl and Bailey, 1957b), and cling peaches (Curl and Bailey, 1959b), minor maxima with N_{100} values of *ca.* 38 were obtained with system I. They were attributed to the presence of monoepoxide monols. In the Meyer lemon pulp there was a minor inflection in this vicinity, which was estimated to be 7% of the total carotenoids. In a 100-transfer run of the peel carotenoids with system I, there was an unusually broad maximum with N_{100} value of 52 and a smaller maximum at N_{100} of 32, both unlike any previously found. In a 200-transfer run, there were three maxima with N_{100} values of 55, 47, and 31 (Fig. 1). The first is close to that of cryptoxanthin (56), but the other two differ from any found previously.

With system IVa the carotenoid diol-polyol fraction was cleanly separated into four fractions in 100 transfers, more cleanly than in 200 transfers with system II (hexane–benzene–87% methanol, Curl 1953). The N_{100} values (Table 1) of these four

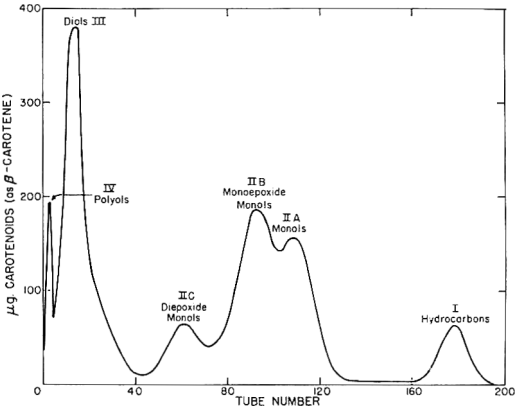


Fig. 1. Countercurrent distribution of Meyer lemon peel carotenoids in system I (hexane–99% methanol).

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

Fraction	Composition (%)		N_{100} value of maxima [a]	
	Peel	Pulp	Peel	Pulp
				(System I)
Hydrocarbon (I)	7	9	90	91
Monol (II or IIA)	18	51	55	57
Monoepoxide monol (IIB)	28	(7) [b]	47	(ca 38) [c]
Diepoxide monol (IIC)	8	.....	31	.....
Diol-polyol (III)	39	33	7	5
				(System IVa)
Diol (IIIA)	6	6	77	76
Monoepoxide diol (IIIB)	3	5	45	45
Diepoxide diol (IIIC)	26	18	17	16
Polyol (IV)	4	3	2	1

[a] N_{100} is the tube number of maximum per 100 transfers.
[b] Approximate value based on inflection at N_{100} of *ca.* 38.
[c] Inflection, estimated.

Table 2. Composition of hydrocarbon carotenoid fraction from Meyer lemons.

Constituent	Spectral absorption maxima, mμ (hexane)	Percentage composition [a]	
		Peel	Pulp
Phytoene	(287),285,273[b]	57	31
Phytofluene	367,348,331[c]	22	20
alpha-Carotene	471,443,417[b]	....	0.5
beta-Carotene	476,449,(427)[b]	2	16
zeta-Carotene	424,399,378[c]	20	32

Values in parentheses are for shoulders or humps on spectral absorption curves.
[a] Calculated from sum of absorbances at principal maximum on Cary spectrophotometer curve for each constituent.
[b] From pulp.
[c] From peel.

fractions were very close to those obtained with the xanthophylls of orange juice and of cling peaches, indicating they consisted of diols, monoepoxide diols, diepoxide diols, and polyols. In the Meyer lemon peel and pulp, the diepoxide diol fraction (IIIC) amounted to over half of the combined diol-polyol fraction (III).

Chromatography. The following fractions were investigated chromatographically; pulp fractions I and II, and peel I, IIA, IIB, and IIC. The other fractions were not chromatographed, because of the small amount of material available and also because the N_{100} values did not indicate anything unusual to be present.

Fraction I (hydrocarbons). The hydrocarbon fractions from both peel and pulp contained considerable amounts of phytoene, phytofluene, and zeta-carotene (Table 2). Beta-carotene occurred in much greater proportion in the pulp than in the peel, and alpha-carotene was found only in the pulp, in very small amount.

Pulp fraction II(monols). The tubes composing IIA and IIB (the minor inflection) were combined for chromatography. Cryptoxanthin amounted to about 88% of the total. It was accompanied by three very minor bands below it on the column, which resembled hydroxy-alpha-carotene or cryptoxanthin-5,6-epoxide, and by two above, which resembled cryptoflavin (cryptoxanthin-5,8-epoxide). The uppermost band had spectral absorption maxima in hexane at 453, 426, and 404 mμ.

Peel fraction IIA (monols). Fractions IIA and IIB (N_{100} values 55 and 47) were incompletely separated, even on a 200-transfer countercurrent distribution run, hence some of the constituents of fraction IIA were found to a small extent in IIB, and vice versa, on chromatography. The constituents are listed in Table 3 under the fractions in which they were mainly found, and the percentages are based on the combined monol fractions IIA, IIB, and IIC.

Much of the color of fraction IIA was due to its high cryptoxanthin content. An unusual feature of this fraction was the presence of two substances not previously found in this laboratory in any fruits. The spectral absorption curves of these fractions closely resembled those of phytoene and phytofluene, but their occurrence in fraction IIA indicated the presence of one hydroxyl group. The chromatographic behavior in relation to cryptoxanthin was very similar to that of phytoene and phytofluene, in relation to beta-carotene. The phytofluenol-like band had a greenish fluorescence on the column in ultraviolet light, similar to that of phytofluene. Zechmeister and Pinckard (1948) reported phytofluenol in some ripe tomatoes, but there appears to be no previous report of phytoenol (hydroxy-phytoene). A hydroxy-zeta-carotene-like pigment was previously found in this laboratory in very small amounts in apricots (Curl, 1960a). Jensen *et al.* (1958) reported the presence of hydroxyphytofluene (phytofluenol) and hydroxy-zeta-carotene in a bacterium, *Rhodospirillum rubrum.*

Peel fraction IIB (monoepoxide monols). The principal constituent of fraction IIB,

Table 3. Composition of monol carotenoid fractions from Meyer lemon peel.

Constituent	Spectral absorption maxima (hexane) (mμ)	Composition %		N_{100} (system I)
IIA				
Phytoenol [a]	298,286	14.0		
Phytofluenol [a]	366,347,330	5.4		
Hydroxy-alpha-carotene [a]	473,444,421	0.8		
Cryptoxanthin	477,449,(426)	22.6		56[b]
Hydroxy-zeta-carotene [a]	425,401,380	2.6		
IIB				
Hydroxy-alpha-carotene 5,6-epoxide [a]	467,440,417	1.2		
Cryptoxanthin 5,6-epoxide	474,445,(422)	27.1		48
Cryptoxanthin 5,8-epoxide a	453,426,404	1.7	3.8	50
Cryptoxanthin 5,8-epoxide b	453,426,403	2.1		
Rubixanthin-like	489,458,431	0.8		
IIC				
Cryptoxanthin 5,6,5′,6′-diepoxide	470,439,416	8.4		32
Phytofluenol-like	366,346,331	0.4		
Cryptoxanthin 5,6,5′,8′-diepoxide a	448,423,400	1.1	2.3	30
Cryptoxanthin 5,6,5′,8′-diepoxide b	448,421,397	1.2		
P378	378,357,340	4.3		27
Cryptoxanthin 5,8,5′,8′-diepoxide	424,398	0.3		31

[a] Tentative identification.
[b] From cling peaches.

which had a spectral absorption curve similar to that of alpha-carotene, was identified as cryptoxanthin-5,6-(or 5′,6′)-monoepoxide. The 5′,6′-monoepoxide was obtained by the action of monoperphthalic acid on cryptoxanthin acetate by Karrer and Jucker (1946). The N_{100} value in system I of the substance obtained in the present work was 48, a rather small difference from that of cryptoxanthin (56). The difference may be due to the epoxide and hydroxyl groups being on the same ring. The substance gave a weak blue color in the hydrochloric acid–ether test. Above it on the column were 2 cryptoflavin-like bands; on countercurrent distribution of the combined bands in system I, the N_{100} value was found to be 50, not significantly different from that of the 5,6-epoxide. Also present were minor bands that appeared to be a hydroxy-alpha-carotene-5,6-monoepoxide and rubixanthin (hydroxy-gamma-carotene); the latter occurred to a somewhat lesser extent in fraction IIA.

The cryptoxanthin-5,6-epoxide fraction was treated 1 min with hydrochloric acid in methanol and ether (1 to 9 to 6 ml) and the product rechromatographed. About 83% of the recovered material consisted of two cryptoflavin-like bands, about 6% of cryptoxanthin. This is about what would be expected if the original substance were cryptoxanthin-5,6-(or 5′,6′)-monoepoxide.

Peel fraction IIC (monol diepoxides). The principal constituent of fraction IIC was identified as cryptoxanthin-5,6,5′,6′-diepoxide. The spectral absorption curve (Fig. 2) was similar to that of violaxanthin (zeaxanthin-5,6,5′,6′-diepoxide). This constituent was accompanied by much smaller amounts of two bands that appeared to be the corresponding 5,6,5′,8′-(or 5′,6′,5,8)-diepoxides, and by a very small amount of the 5,8,5′,8′-diepoxide. The 5,6,5′,6′- and 5,6,5′,8′-diepoxides gave a light blue color in the hydrochloric acid–ether test.

The 5,6,5′,6′-diepoxide was treated 2 min with hydrochloric acid in methanol (1:9),

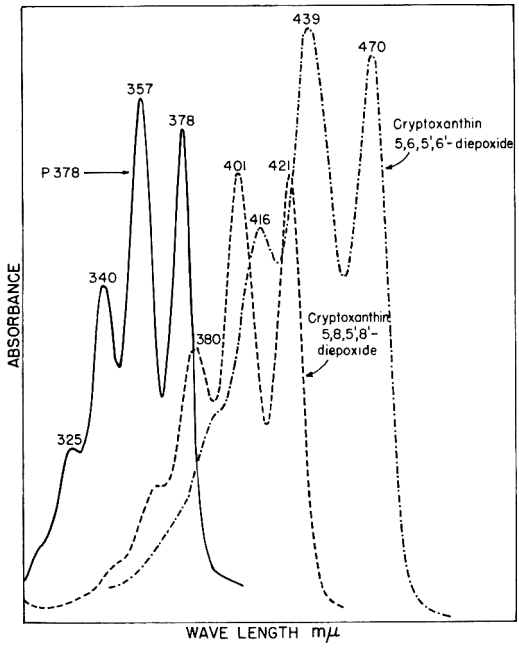


Fig. 2. Spectrophotometric curves in hexane of carotenoids from Meyer lemon peel.

and the product chromatographed. The 5,8,5′,8′-diepoxides amounted to 75% of the recovered material, with about 10% of cryptoflavin-like substances, which supports the identification of the original substance. The spectral absorption curve of the 5,8,5′,8′-diepoxide (Fig. 2) closely resembles that of auroxanthin (zeaxanthin-5,8,5′,8′-diepoxide). The N_{100} values (Table 3) of the 3 diepoxides were 30, 31, and 32.

Above the 5,6,5′,8′-diepoxide bands on the column was a substance with spectral absorption maxima at 378, 357, and 340 mμ (P378) in hexane (Fig. 2). The shape of the curve resembled that of a carotenoid with none of the conjugated double bond system in an ionone ring, such as phytofluene. The spectral absorption maxima were at about 11, 9, and 7 mμ, respectively, longer wavelengths than those of phytofluene, which has a system of 5 conjugated double bonds. No greenish fluorescence in ultraviolet light was observed. The N_{100} value in system I was 27, a little below that of cryptoxanthin-5,6,5′,6′-diepoxide, but much above that of the diols lutein (10) and isozeaxanthin (15).

In order to test for the presence of epoxide groups, a portion of the sample was treated 15 sec with hydrochloric acid in methanol (1:9). The spectral absorption maxima were now at 398, 376, and 356 mμ (in hexane), respective increases of 20, 19, and 16 mμ, which indicates the addition of a double bond to the conjugated system instead of the loss that occurs with a 5, 6-epoxide.

The acid-treated product was subjected to a 100-transfer countercurrent distribution run with system I. The distribution curve showed the presence of two well-defined maxima with N_{100} values of 63 and 86; the latter was about twice as great in amount as the former. These two substances had almost identical spectral absorption curves and maxima; the shape of the curves were similar to those of the original material.

The N_{100} value of 63 is somewhat above that of cryptoxanthin at 56 and may indicate one hydroxyl group remaining. The shift in N_{100} value from 27 to 63 may indicate the loss of one hydroxyl group. This can be accounted for by assuming that one hydroxyl group originally was allylic to one end of the conjugated double bond system. Upon hydrochloric acid treatment a molecule of water was lost, thus an additional double bond was formed.

The N_{100} value of 86 is somewhat below that of beta-carotene, which is 91, and may indicate the presence of one methoxyl group only. The substance with N_{100} of 86 may be the methyl ether of the substance with N_{100} of 63. This may also indicate that the second hydroxyl group is also allylic, too, perhaps to an isolated double bond. It was shown by Petracek and Zechmeister (1956) that some allylic hydroxyl groups in carotenoids will form methyl ethers on treatment with acidic methanol.

It can be concluded that P378 is a polyene that contains a conjugated double bond system of about 5 double bonds, a hydroxyl group allylic to the conjugated system, and probably another hydroxyl group that is also allylic but perhaps not to the conjugated system. This polyene may not be a carotenoid or a polyene with a related structure, such as phytofluene.

ACKNOWLEDGMENT

Spectral absorption data were obtained by Glen F. Bailey and Edith L. Long. The writer is indebted to the late G. F. Siemers, Hoffmann-LaRoche, Inc., Nutley, N. J., for the sample of isozeaxanthin.

REFERENCES

Curl, A. L. 1953. Application of countercurrent distribution to Valencia orange juice carotenoids. *J. Agr. Food Chem.* **1**, 456.

Curl, A. L. 1960a. The carotenoids of apricots. *Food Research* **25**, 190.

Curl, A. L. 1960b. Two new solvent systems for the countercurrent distribution of carotenoids. *J. Agr. Food Chem.* **8**, 356.

Curl, A. L., and G. F. Bailey. 1954. Polyoxygen carotenoids of Valencia orange juice. *J. Agr. Food Chem.* **2**, 685.

Curl, A. L., and G. F. Bailey. 1955. The state of combination of the carotenoids of Valencia orange juice. *Food Research* **20**, 371.

Curl, A. L., and G. F. Bailey. 1956. Comparison of carotenoids of Valencia orange peel and pulp. *J. Agr. Food Chem.* **4**, 156.

Curl, A. L., and G. F. Bailey. 1957a. The carotenoids of Ruby Red grapefruit. *Food Research* **22**, 63.

Curl, A. L., and G. F. Bailey. 1957b. The carotenoids of tangerines. *J. Agr. Food Chem.* **5**, 605.

Curl, A. L., and G. F. Bailey. 1959a. Changes in the carotenoid pigments in preparation and storage of Valencia orange juice powder. *Food Technol.* **13**, 394.

Curl, A. L., and G. F. Bailey. 1959b. The carotenoids of cling peaches. *Food Research* **24**, 413.

Curl, A. L., and G. F. Bailey. 1961. The carotenoids of navel oranges. *J. Food Sci.* **26**, 442.

Jensen, S. L., G. Cohen-Bazire, T. O. M. Nakayama, and R. Y. Stanier. 1958. The path of carotenoid synthesis in a photosynthetic bacterium. *Biochim. biophys. acta.* **29**, 477.

Karrer, P., and E. Jucker. 1946. Vom Kryptoxanthin sich ableitende Epoxyde and furanoide Oxyde. *Helv. Chim. Acta.* **29**, 229.

Petracek, F. J., and L. Zechmeister. 1956. Reaction of beta-carotene with N-bromosuccinimide: the formation and conversions of some poly ketones. *J. Am. Chem. Soc.* **78**, 1427.

Zechmeister, L., and J. H. Pinckard. 1948. On naturally occurring colorless polyenes. *Experientia* **4**, 474.

The Browning Produced by Heating Fresh Pork. I. The Relation of Browning Intensity to Chemical Constituents and pH[a,b]

A. M. PEARSON, G. HARRINGTON,[c] R. G. WEST,[d] AND MILDRED E. SPOONER

Department of Food Science,[e] Michigan Agricultural Experiment Station, East Lansing, Michigan

SUMMARY

A relationship between the degree of brownness produced by deep-fat frying or by evaporating to dryness in a 100°C electric oven was established. Fresh pork as chops, in a water slurry, or a water extract developed varying degrees of brownness on heating. The amount of brown color development was related to the level of reducing sugars in the tissues. The degree of color development could be measured spectrophotometrically at a wavelength of 375 mμ. The development of brownness in buffered solutions was pH-dependent, with maximum color being produced between pH 5.60 and 5.90.

INTRODUCTION

The browning of certain foods during cooking is recognized to impart attractiveness and to add a desirable undertone to the natural flavors. The importance of brownness as related to the flavor and appearance of meat and some other foods was recognized early by the manufacturers of microwave cooking equipment, who commonly recommended pre-browning of foods so cooked. The exact mechanism whereby browning produces desirable flavors is not known.

Observations in our laboratory showed that raw pork from different animals varied in degree of brownness when subjected to deep-fat frying or to oven drying under standard conditions. No information or observations on this phenomenon were noted in a literature search, so this study was initiated to determine the relationship between the constituents of pork muscle and the development of brown color upon heating.

PROCEDURE

Pork—origin. Pork loin chops were removed from the center portion of the right loin of 10 different hogs selected at random from the regular pork kill at the University abattoir. Although the hogs came from various treatments at the University farm, standardized feeding, holding, slaughtering and chilling procedures were followed in the laboratory. After slaughter, the carcasses were chilled for 48 hr at approx 2°C before being cut. The loins were removed, cut, and frozen until used.

Browning and Ranking. A center section of the loin from each hog was ground, and a sample of each was heated 24 hr at 100°C in disposable aluminum weighing dishes in a forced-air electric drying oven. The samples were cooled and ranked visually in order of brownness from 1 through 10, from lightest to darkest. In addition, chops from each loin were fried in lard heated to 138°C until the internal temperature reached 80°C. They were removed, cooled, and ranked visually in order of brownness as with the oven-dried samples.

Chemical analysis. Further samples from each loin were prepared for analysis of the reducing sugars in the following manner: One g of ground

---

[a] Journal Article 2700, Michigan Agricultural Experiment Station, East Lansing.

[b] The authors thank the W. K. Kellogg Foundation, Battle Creek, Michigan, for support of this research, in the form of a fellowship for G. Harrington and other financial assistance.

[c] Present address: Agricultural Research Council, Statistics Group, School of Agriculture, Cambridge University, Cambridge, England.

[d] Deceased.

[e] The authors gratefully acknowledge the assistance of Dr. J. R. Brunner, Department of Food Science, for recording the absorption spectra of the samples.

loin tissue was dispersed in 10.5 ml of H_2O; 6.4 ml of 0.2091*N* H_2SO_4 was then added, and the mixture was allowed to stand for 2 min. The sugar content of the muscle was determined by the method of Folin and Wu (1920) and expressed in terms of glucose. Fat, protein, and moisture were determined by the methods of Benne *et al.* (1956), and pH readings were taken on a meat-water slurry (1 g meat in 10 ml of water) with a Model G Beckman pH meter. Free amino nitrogen was determined for the same meat-water slurry by the method of Van Slyke as outlined in the A.O.A.C. (1955) and was expressed as percentage of free amino nitrogen in the tissues. Glycogen was determined by the method of Hansen *et al.* (1952), and lactic acid was measured colorimetrically by the method of Barker and Summerson (1941).

Objective measurement of brownness. To ascertain whether absorbance could be used to measure development of brownness, a 1:3 meat-water slurry was prepared in a Waring blender from each raw pork loin. Ten-ml portions were removed from each slurry and combined with 10 ml of distilled water. All samples were then dried for 24 hr to develop the brown color, resuspended in 20 ml of distilled water, using a small spatula to break up the particles, and filtered through Whatman 41 filter paper. Two ml of the filtrate was diluted to 20 ml with distilled water, and the solution was used for reading the absorption spectrum.

Absorbance curves were plotted from readings at wavelengths of 350, 375, 400, 425, and 450 mμ with a Bausch and Lomb Spectronic 20 colorimeter. In addition, spectral curves were plotted for several of the browned samples with the Beckman DK-2 recording spectrophotometer.

Buffers. To determine whether pH influences the development of brown color, a series of phosphate buffers were prepared by using 1*M* KH_2PO_4 and 2*M* K_2HPO_4 in proportions to give pH values of 5.5, 6.0, 6.5, 7.0, and 8.0. Ten-ml portions of each 1:3 meat-water slurry were added to 10 ml of each buffer, so that samples of each loin were buffered at each pH. In addition, one aliquot of the meat-water slurry from each loin was added to 10 ml of distilled water. All samples were checked for pH, dried to develop brownness, resuspended in water, and filtered, and absorbance was measured with the Spectronic 20 colorimeter as outlined previously.

Statistical analysis. The rankings for brownness were correlated with the objective color measurements by Spearman's ρ as outlined by Kendall (1955). Otherwise, usual correlation methods were used.

RESULTS AND DISCUSSION

Development of brownness on heating. Both the deep-fat-fried chops and the oven-dried samples showed considerable variation in brownness. It was found on drying replicate samples that they maintained about the same relative rank. Some samples were a burnt-brown and others a light-brown. Thus, it was possible to make fairly distinct differentiation in color rankings. Although observations leading to this study were made on deep-fat-fried chops, it became apparent that color development was not only darker but more consistently reproducible for the oven-dried samples. The correlation in ranking between the two methods of browning for these 10 loins was 0.77. Since color development tended to be more reproducible for the oven-dried samples, they were used as the basis for other comparisons.

Measurement of brownness by absorption spectra. Subjective ranking according to color intensity is at best difficult, so absorption spectroscopy was investigated as a possible objective measure of degree of brownness. Fig. 1 shows a characteristic absorption spectrum of an aqueous solution of the browned meat extract made with the Beckman DK-2 recording spectrophotometer. Optical density increased as wavelength was decreased. The curve shows the regularity of the increase in absorbance as wavelength was decreased. Since the colored extracts showed good absorbance at 375 mμ and readings could be readily made with the Spectronic 20 colorimeter, this wavelength was arbitrarily selected as a point for measuring color development. Color readings made at 375 mμ are given in Table 1. These show that the rank of the 10 loins according to the objective color measurements had a correlation of 0.70 with the ranks of the fried chops and of 0.95 with the ranks of the oven-dried samples according to apparent brownness. This indicates that absorbance was an excellent measure of brownness in the oven-dried samples. Although no extracts were prepared of the deep-fat-fried chops, the close relation between the color rankings for fried chops and the oven-dried samples indicates that absorbance could probably also be used to measure the

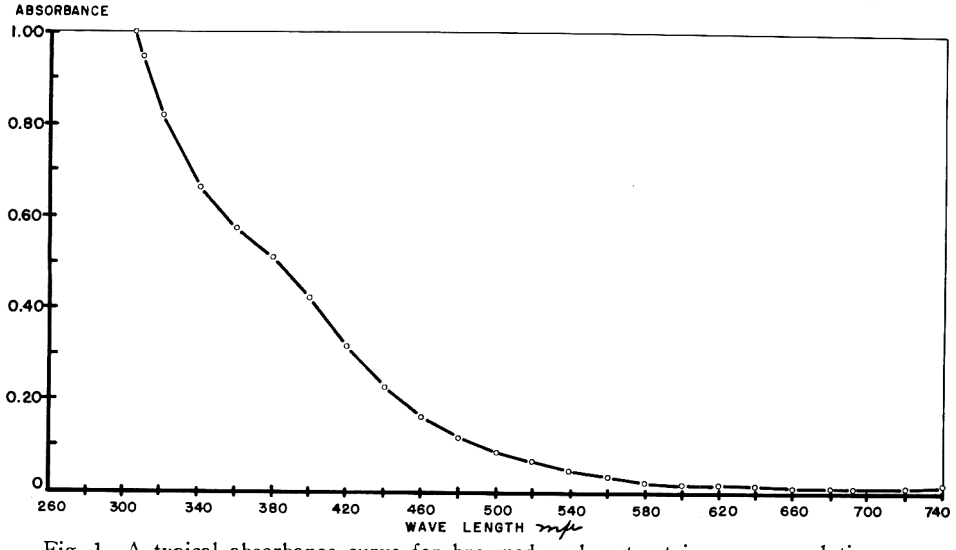


Fig. 1. A typical absorbance curve for browned pork extract in aqueous solution.

intensity of brownness for the fried chops.

Measurement of absorbance in the manner described appears to be a valid objective method of expressing the degree of brownness developed on heating fresh pork. Once the nature of the reaction is established, development of standard procedures for measuring the amount of brownness produced on heating meat or meat extracts should be possible.

Relationship of chemical constituents to color development. Table 2 summarizes the data on objective color measurements and various chemical components. Examination of the data reveals that the level of sugar in the tissues was closely related to color development with a correlation of 0.97 (Table 3). The significant negative relationship ($r = -0.66$) between percentage protein and color development was unexpected. The correlation, however, was influenced to a considerable extent by the two darkest-colored loins (Nos. 82-1 and 82-13), which were lowest in protein content.

The extremely high relationship between free sugar content and degree of brownness suggests that sugar is, at least in part, responsible for the brown color developed upon heating. This would indicate that the color production may be due to the amino-sugar (Maillard) reaction, which is known to produce an undesirable browning in a number of foods (Ellis, 1959). Although the amino-sugar reaction has not been

Table 1. Values and relative ranks for brownness and absorption spectra for pork loins.

Pig No.	Rank for oven-dried samples[1a]	Rank for deep-fat-fried chops[1a]	Absorption spectra values at 375 mμ	Rank for absorption spectra values[1a]
86–11	1	2	0.090	1
86-9	2	3	0.096	2
27–3	3	1	0.108	3
23–17	4	4	0.163	6
29–4	5	8	0.142	5
27–1	6	7	0.120	4
23–11	7	5	0.221	7
82–6	8	9	0.290	8
82–13	9	10	0.486	9
82–1	10	6	0.579	10

[a] Rankings are from 1 to 10, from lightest to darkest.

Table 2. Absorption spectra and chemical constituents in different pork loin samples.

Pig No.	Absorption spectra values at 375 mμ	Reducing sugar as glucose (mg %)	Glycogen (mg/g)	Lactic acid (mg %)	pH	Percent free amino nitrogen	Percentage Fat	Protein	Moisture
86–11	0.090	57	0.04	760	6.10	0.21	5.76	20.84	71.74
86–9	0.096	68	0.09	780	5.88	0.17	5.62	20.71	72.44
27–3	0.108	113	0.68	920	5.70	0.28	2.15	21.92	73.81
23–17	0.163	222	2.56	950	5.52	0.16	2.65	21.48	74.03
29–4	0.142	210	1.33	970	5.77	0.26	3.79	22.38	72.95
27–1	0.120	273	1.54	1010	5.62	0.24	2.72	22.75	73.43
23–11	0.221	387	4.59	930	5.35	0.27	4.70	21.78	72.00
82–6	0.290	436	0.04	1040	5.62	0.24	2.14	22.47	73.46
82–13	0.480	752	0.05	950	5.62	0.19	8.52	19.34	70.63
82–1	0.579	763	0.05	930	5.58	0.23	4.17	19.64	74.05

studied in development of the desirable brown color in cooked meats, its implication in the browning of stored dehydrated meats has been shown by a number of workers (Regier and Tappel, 1956; Tappel, 1956; Sharp, 1957 a, b). Henrickson *et al.* (1955) showed that the browning of dehydrated meats could be largely prevented by yeast fermentation of the sugars. Another possible mechanism for the development of the brown color in cooked meats may be by caramelization of the naturally occurring sugars, for this reaction has been demonstrated in other products by Zerban (1947). Browning on heating fresh pork may also be due to a combination of caramelization and the amino-sugar reaction. Additional work has been initiated to elucidate the exact mechanism of the brown color development obtained upon heating fresh pork.

Influence of pH on color Development. Fig. 2 shows absorbance values for the buffered meat extracts following browning. The absorption curves are plotted so that one is based on pH readings taken before browning, and the other on values obtained after the sample had been browned and rehydrated. The two curves closely parallel each other except in the unbuffered range, where the absorption peak occurred at approximately pH 5.75 for the browned-rehydrated sample, compared with a pH of 5.90 for the value before browning. It is interesting to note that the peak absorption occurred in the unbuffered sample, and that browning resulted in a definite alteration in pH in the absence of the buffer but remained essentially the same for the buffered samples.

Development of brown color was maximum somewhere in a pH range of 5.60–5.90 (Fig. 2). The darkest-colored samples

Table 3. Correlation coefficients for absorption spectra at 375 mμ with various chemical components in pork loin samples.

	"r"
Reducing sugars (as glucose)	0.97**
Glycogen level	−0.24
Lactic acid	0.31
pH	−0.41
Free amino nitrogen, %	−0.06
Fat, %	0.35
Protein, %	−0.66*
Moisture, %	−0.09

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

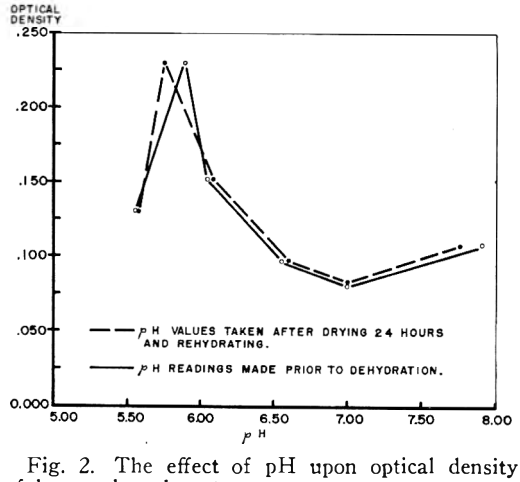


Fig. 2. The effect of pH upon optical density of browned pork extracts.

occurred at pH 5.60 (Table 2), but in a heterogeneous product such as meat there would obviously be some variation in the optimum. Since phosphate buffers were added to raise the pH in this study, it is possible that the addition may have inhibited browning. However, studies by Willits *et al.* (1958) and Underwood *et al.* (1959) showed that an acid pH tends to inhibit browning and that it is necessary to raise the pH to the alkaline side of neutral in order to obtain maximum color development. Therefore, it seems unlikely that the increase in browning noted on heating meat in a pH range of 5.60–5.90 was influenced by the phosphate additions.

REFERENCES

A.O.A.C. 1955. "Official Methods of Analysis." 8th ed. Association of Official Agricultural Chemists. Washington, D. C., p. 390.

Barker, S. B., and W. H. Summerson. 1941. The colorimetric determination of lactic acid in biological material. *J. Biol. Chem.* **138**, 535.

Benne, E. J., N. H. Van Hall, and A. M. Pearson. 1956. Analysis of fresh meat. *J. Assoc. Offic. Agr. Chemists.* **39**, 937.

Burnett, M. C., C. W. Gehrke, and D. E. Brady. 1955. Dehydrated meat. Volatile components of vacuum packed dehydrated pork. *J. Agr. Food Chem.* **3**, 524.

Ellis, G. P. 1959. The Maillard reaction. *Adv. Carbohydrate Chem.* **14**, 63.

Folin, A., and H. Wu. 1920. A simplified and improved method for determination of sugar. *J. Biol. Chem.* **41**, 367.

Hansen, R. G., W. G. Rutter, and E. M. Crowe. 1952. A nephelometric method for determination of glycogen. *J. Biol. Chem.* **195**, 127.

Henrickson, R. L., D. E. Brady, C. W. Gehrke, and R. F. Brooks. 1955. Dehydrated pork studies. Removal of glucose by yeast fermentation. *Food Technol.* **9**, 290.

Kendall, M. G. 1955. "Rank Correlation Methods." p. 8, 2nd ed. Charles Griffin and Co. Ltd., London.

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

Sharp, J. G. 1957a. Deterioration of dehydrated meat during storage. I. Nonenzymic deterioration in absence of oxygen at tropical temperatures. *J. Sci. Food Agr.* **8**, 14.

Sharp, J. G. 1957b. Deterioration of dehydrated meat during storage. II. Effect of pH and temperature on browning changes in dehydrated aqueous extracts. *J. Sci. Food Agr.* **8**, 21.

Tappel, A. L. 1956. Freeze-dried meat. II. The mechanism of oxidative deterioration of freeze-dried meat. *Food Research* **21**, 195.

Underwood, J. C., H. G. Lento, Jr., and C. O. Willits. 1959. Browning of sugar solutions. 3. Effect of pH on the color produced in dilute glucose solutions containing amino acids with the amino group in different positions in the molecule. *Food Research* **24**, 181.

Willits, C. O., J. C. Underwood, H. G. Lento, Jr., and C. Rissiuti. 1958. Browning of sugar solutions. I. Effect of pH and type of amino acid in dilute sugar solutions. *Food Research* **23**, 61.

Zerban, F. W. 1947. The color problem in sucrose manufacture. Sugar Research Foundation Report 2. Sugar Research Found., New York, p. 1.

Automatic Recording of Vibrational Properties of Foodstuffs

BIRGER DRAKE
Swedish Institute for Food Preservation Research, Götcborg, Sweden

(Manuscript received May 10, 1961)

SUMMARY

An apparatus is described for automatic recording of mechanical resonance curves for test specimens of foodstuffs with the approximate size of 6 × 12 × 50 mm. The simple evaluation procedure described gives information on the modulus of elasticity (divided by the density) and degree of mechanical damping.

In judging the consistency of solid and semisolid foodstuffs, vibrational properties are often, consciously or unconsciously, estimated as an important part of the general mechanical properties. Thus, for a better understanding of the relations between subjective and objective determinations, a study of vibrational properties fills a given place. This is especially so since vibration measurements are in some respects easier to perform than static ones, and it is usually not necessary to know the absolute magnitude of the amplitude of vibration.

Various manual and automatic measurements of vibrational properties of engineering materials can nowadays be considered a well established procedure (Bopp *et al.*, 1947; Bradfield, 1960; Grime and Eaton, 1937; Kohlrausch, 1955; Moyal and Fletcher, 1945). Usually, however, foodstuffs and other materials of biological origin have been investigated by point-for-point methods (Anon., 1954; Brooks and Hale, 1959; Kärrholm and Schröder, 1953; Virgin, 1955) and only occasionally with some sort of automatic recording (Shimizu and Ichiba, 1958). It was thus deemed suitable to design an automatic apparatus specially for such materials, which are quite different from more frequently investigated materials, such as metals. The alternative chosen here was to record automatically the mechanical resonance curves for transversally vibrating cantilevers. Such curves can easily be evaluated manually as to mechanical damping and, when geometric dimensions are exactly known, modulus of elasticity.

Theory. An appropriate treatment of the theory of mechanical vibrations can be found in textbooks (Hartog, 1947; Kohlrausch, 1955; Santen, 1953; Schuler, 1958), and only some minor extensions had to be developed for the purpose considered here.

For a forced harmonic vibration, the following formula is applicable:

$$m\ddot{x} + r\dot{x} + kx = P\cos\omega t \qquad [1]$$

where m = mass of the oscillator,
r = "damping constant" (related to viscosity),
k = "spring constant" (related to elasticity),
P = peak value of sinusoidal external force,
x = deviation (points indicate time derivatives),
ω = angular frequency = $2\pi f$ (where f = frequency),
t = time.

The steady-state solution of this equation may be written

$$x = \frac{A_o \cos(\omega t - \phi)}{\sqrt{(1-n^2)^2 + n^2\delta^2}} \qquad [2]$$

where $A_o = P/k$ = constant deviation for a static force of magnitude P,
ϕ = phase angle between force and deviation = arc tan $[\omega r/(k - \omega^2 m)]$
n = relative frequency (f/f_o or $\omega/\omega_o = \omega/\sqrt{k/m}$ (where ω_o = resonance frequency for the same oscillator without damping),
δ = "damping factor" = $r/\sqrt{k \cdot m}$.

The amplitude A of the vibration (i.e., the maximum deviation of the oscillating body) may then be written as

$$A = A_o / \sqrt{(1-n^2)^2 + n^2 \delta^2}. \qquad [3]$$

The quotient A/A_o, often referred to as "amplification factor" R, has a maximum value $R_{max} = 1/\delta\sqrt{1 - \delta^2/4}$, when n has the resonance value $n_r = \sqrt{1 - \delta^2/2}$. Obviously, for small δ values R_{max} will be very high and n_r close to 1, while for increasing δ values both R_{max} and n_r will decrease (Fig. 1). For $\delta < 0.2$, the difference between n_r and 1 (and also between observed resonance frequency and "ideal resonance frequency" f_o) is smaller than 1%, whereas for $\delta < 0.4$ it is smaller than 4%.

EXPERIMENTAL

Apparatus. In principle, the recording of resonance peaks for a test specimen demands: 1) a frequency sweep, 2) a mechanical driving action, 3) a frequency measurement, 4) an amplitude measurement, and 5) a recording of these two measurements simultaneously. In the actual case considered here, the five needs are fulfilled by using: 1) a low-frequency generator with a motor connected to the double potentiometer of a Wien-bridge arrangement, 2) a transistor amplifier feeding two electromagnets acting on a small piece of iron inserted into the specimen, 3) a conventional frequency meter with the same ranges as the low-frequency generator, 4) a lamp/lens/slit/phototube/amplifier arrangement for measuring the amplitude of vibration (*cf.* Sack *et al.*, 1947), and 5) an X-Y-recorder (Mandrel, Type ER-90) that draws on a sheet of paper a curve of amplitude as a function of frequency.

A block diagram of the complete apparatus is shown in Fig. 2, and some details in Fig. 3. Also,

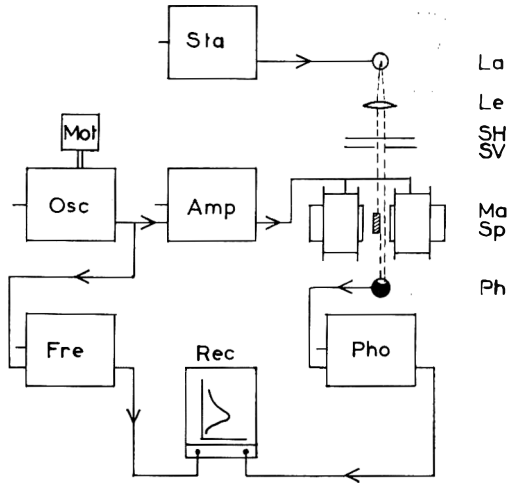


Fig. 2. Block diagram of the apparatus: Sta = stabilizer for the lamp; Mot = sweep motor; Osc = low frequency oscillator; Amp = amplifier for the electromagnets; Fre = frequency meter; Rec = X–Y recorder; Pho = phototube amplifier; La = lamp; Le = lens; SH = horizontal slit; SV = vertical slit; Ma = 2 electromagnets; Sp = test specimen; Ph = phototube.

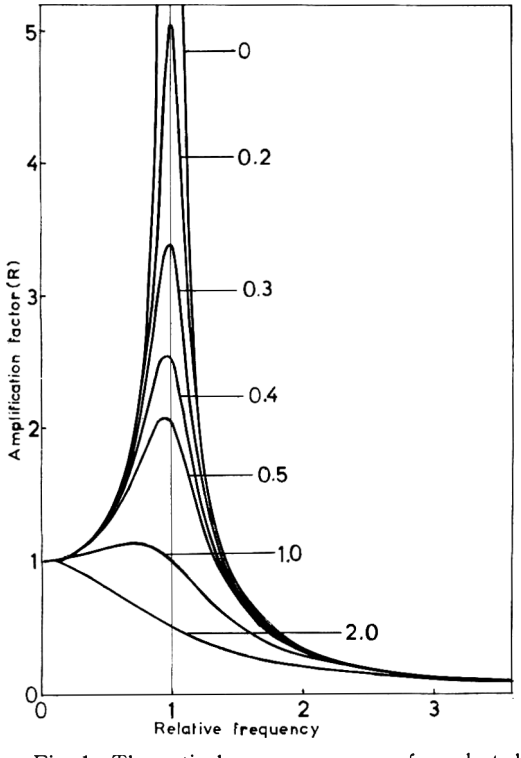


Fig. 1. Theoretical resonance curves for selected damping-factor values.

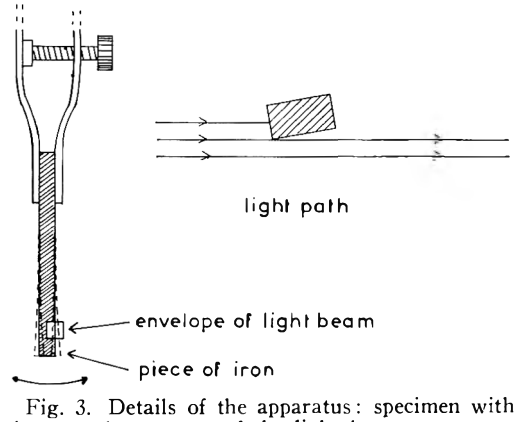


Fig. 3. Details of the apparatus: specimen with clamp, and geometry of the light beam.

two curves obtained with the apparatus for a relatively hard foam rubber used for adjustment purposes are shown in unretouched form in Fig. 4.

So that the frequencies relevant to the measurement on foodstuff specimens of reasonable size are included, the generator was built for the total range approx 1 c/s to 5000 c/s, divided into subranges whose upper limits are placed at 2, 5, 10, 20, etc., c/s. Each one of the subranges covers the frequencies from approx 20% to 100% of the upper limit.

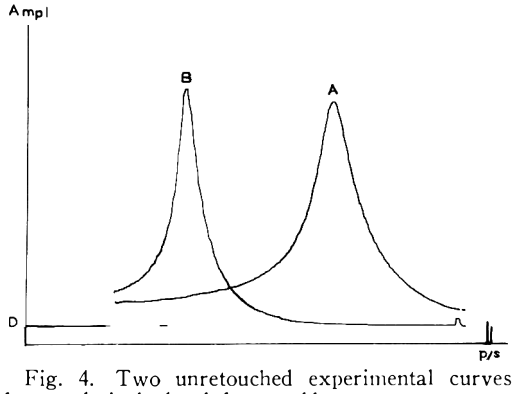


Fig. 4. Two unretouched experimental curves for a relatively hard foam rubber.

At lower frequencies, the damping of the rectified output from the frequency meter and from the amplitude meter becomes a critical question. Thus, for satisfactory results with the present arrangement, the usual recording time of 5 min should be increased with frequencies lower than about 5–10 c/s. Otherwise, even if the curves look satisfactory, too much delay (by using large capacitances for smoothing purposes) will cause trouble with accurate reading of both frequency and amplitude.

To utilize the full capacity of the apparatus outlined here, two conditions should be satisfied: One needs a suitable method of cutting foodstuffs into exact geometrical shapes, and also a suitable method of evaluating experimental curves. How these separate problems were solved is described below.

Accessory arrangements. At an early stage, it was found impossible to cut exactly rectangular test specimens from soft materials by using, e.g., a set of two knives or two safety-razor blades. Later, a cutting arrangement was developed that has given more satisfactory results.

The cutting is now performed in two stages. First, a combination of two thin parallel knives, hinged at one end, is turned down over a suitable piece of the material, and the operation is repeated after the cut-out part is rotated 90°. The square rod (17 × 17 mm) obtained in this way is placed in a tight-fitting aluminum box provided with a set of longitudinal slots (Fig. 5), and then covered with a plastic plate. Finally, the shaping is completed with rotating sawblades in three cuttings, two with double-blades and one with a single blade. As a result, two test specimens with (in our case) approx 5.5 and 7 mm thickness and 12 mm width are obtained, together with a number of surrounding strips, which are discarded.

Evaluation of recorded curves. A vertical line is drawn through the vertex, peak height H (Fig. 6), and a horizontal line is drawn at a height of $h = H \cdot 0.707$ $(= H \sqrt{2})$. H and H_0 are used here instead of A and A_0 to indicate height over the base line on the graph paper. (Since the present model of the apparatus has not yet been made completely screened, noise and hum in the electrical circuits give a certain dark current reading. For very low peaks, this gives an appreciable deformation of the peak shape. For the curves shown in this article, however, the correction for the upper half of the peak is almost negligible.) The width Δn of the peak cut off by this line, and the distance n_r between the vertical line and the line $n = 0$, are measured in millimeters and tenths thereof. The quotient $\Delta n/n_r$ (which equals $\Delta f/f_r = \Delta\omega/\omega_r$) gives a measure of peak width and is, for low degrees of damping, equal to the *damping factor* δ. For higher damping fac-

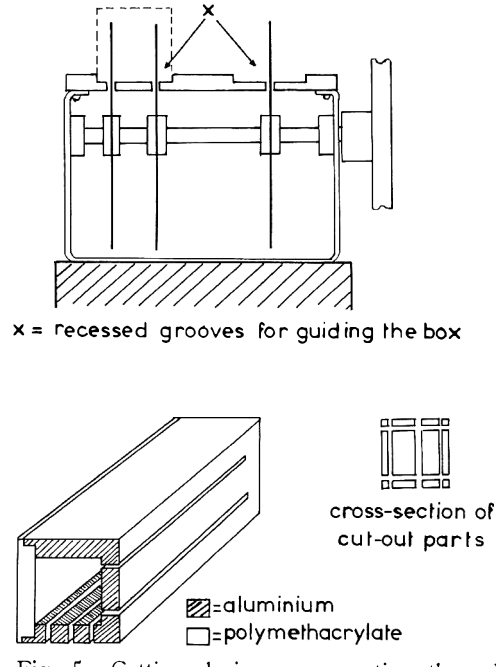


Fig. 5. Cutting device: cross-section through rotating saw, and sketch of special aluminum box.

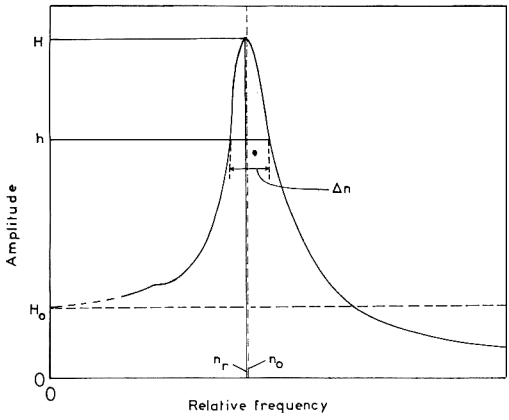


Fig. 6. Evaluation of a (smoothed) experimental curve for a soft foam rubber. (Full lines are needed for estimation of $\Delta n/n_r$; dashed lines for $f = f_o$ and $H = H_o$ have been drawn for control purposes.)

tors, however, a more complicated relation has to be used, e.g.,

$$\Delta n/n_r = \sqrt{1+\alpha} - \sqrt{1-\alpha},$$

where $\alpha = \delta \sqrt{1-\delta^2/4} \; / \; (1-\delta^2/2)$. [4]

From calculations leading to the curve shown in Fig. 7, which by the aid of equation 4 gives $\Delta n/n_r$ as a function of δ, it has been found that the error in using the simple relation $\Delta n/n_r = \delta$ is smaller than 2% for values of δ below approx 0.2. However, the difference will evidently become appreciable for higher δ values, such as are not uncommon among foodstuffs.

For the foam rubber used in the experiments that gave the curves in Fig. 4, three consecutive determinations of $\Delta n/n_r$ have given respective values 0.117, 0.113, and 0.113, whereas in runs performed on various other days, the values were 0.122, 0.112, and 0.111. This gives a rough estimate of the degree of accuracy that can be obtained with the actual model.

For the determination of *modulus of elasticity* of a beam with rectangular cross-section, the following formula (Kohlrausch, 1955) may be used.

$$E = 48\,\pi^2 \rho l^4 f^2 / m^4 a^2. \qquad [5]$$

Here ρ = density, l = length, F = frequency, m = a factor with certain "eigenvalues" for the different modes of vibration, and a = thickness of the beam. Since $m^4 = 12.36$ for the fundamental frequency, the following formula can be applied:

$$E = 38.3\,\rho\, l^4 f^2 / a^2 = 38.3\,\rho\,\left(\frac{l^2}{a} \cdot f\right)^2 \qquad [6]$$

It should be remembered that, since the test specimens used have been relatively short, formulas 5 and 6 are only approximately valid. For foodstuffs with a high content of water the density is close to unity, and the formulas then become even simpler. In this first report, however, only relative values were aimed at, and the coefficients of these formulas are consequently not considered important.

The fact that l here occurs in the fourth power, indicates that determination of test-specimen length is rather critical. Thus, e.g., a misreading of 0.5 mm for a length of 40 mm gives an error in E of 5%. On the other hand, an equal misreading of 0.5 mm for a width of 5 mm gives an error in E of 20%, which shows that great care is necessary in measuring specimen width. It is also evident that uniformity of specimen width must be an important factor.

As expected, a comparison of values obtained for modulus of elasticity has shown that different lengths of the same test specimen do not give the same result. However, the addition of a length equal to the specimen's thickness to all lengths measured gives a practically constant E value, and such a correction has accordingly been applied here. (A closer study of this phenomenon will be taken up later.)

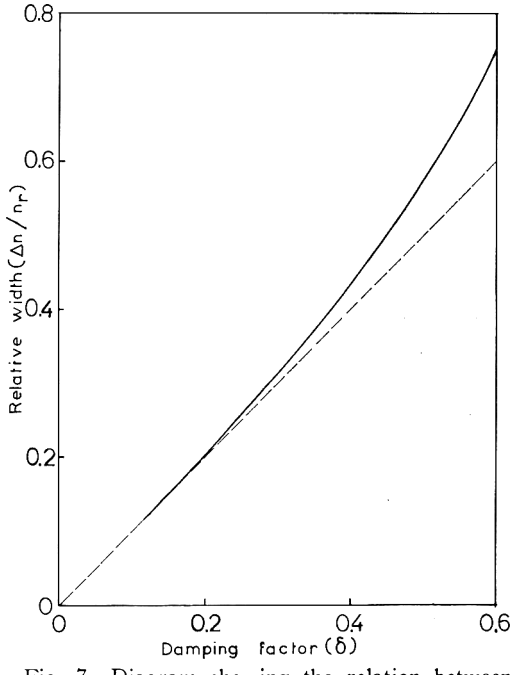


Fig. 7. Diagram showing the relation between relative peak width $\Delta n/n_r = \sqrt{1+\alpha} - \sqrt{1-\alpha}$, where $\alpha = \delta\sqrt{1-\delta^2/4} \; / (1-\delta^2/2)$, and the damping factor δ.

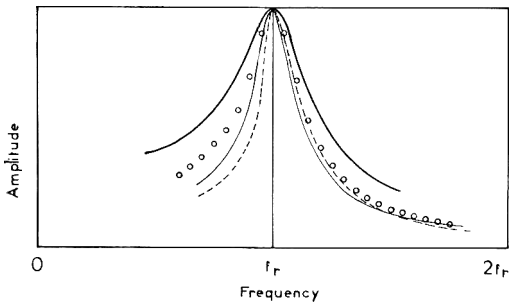


Fig. 8. Comparison between (smoothed) experimental curves for potato (————), pear (– – –), and cheese (———), which before the recording have been adjusted so that the vertices coincide on the graph paper. Theoretical values for $\delta = 0.2$ have been inserted (o).

To simplify comparisons between curves for different materials or different specimens of the same material, it can be advantageous to adjust the amplifications in the two channels connected to the X–Y recorder in such a way that all vertices fall exactly on a point with, e.g., the coordinates $f_r = 10$ cm and $H = 10$ cm. An example of such a comparison is given in Fig. 8. Here, the damping factors for the 3 specimens can be easily compared and measured with the same degree of accuracy, while the calculation of E values demands a knowledge of the actual amplification along the frequency axis.

Measurements on some foodstuffs. In order to test the performance of the apparatus described here, a number of foodstuffs have been cut into test specimens and run at room temperature in an appropriate frequency range. The curves obtained have then been evaluated, in the way described above, as to damping and modulus of elasticity. This has usually involved no trouble, but in some cases where the properties investigated have had very high or very low values, the results may have been less accurate than in the most suitable range.

Information on some of the materials investigated is in Table 1, which gives experimental data and characteristic values calculated from them. It should be observed that the values (especially E values) change with time after the moment of cutting, and that reliable values will accordingly demand a series of measurements and an extrapolation to the time of cutting. An idea about both the accuracy in measuring and the changes with time can be obtained from Figs. 9 and 10. As a preliminary result, it can be observed that E values for cheese and fish pudding increase with time after cutting, whereas the corresponding values for apple, pear, and potato decrease. As to possible evaporation effects, these can be largely avoided by, e.g., surrounding the specimen with a transparent box (a possibility elaborated on later

Table 1. Experimental data and characteristic values for some selected foodstuffs (specimen width perpendicular to vibration direction, 1.2 cm).

Material	a (cm)	l (cm)	f_r c/s	E/ρ (rel. values)	δ
Apple	0.7	4.3	36.6	156	0.075
("Jonathan")	0.6	4.8	21.2	100	0.08
Fish pudding (commercial)	0.6	4.9	5.8	8.5	0.13
Potato (commercial)	0.6	5.3	15.0	76	0.17
Pear ("Pecknam")	0.55	4.4	18.8	71	0.195
Cheese I	0.6	4.8	15.8	55	0.34
("Herrgård")	0.6	5.8	14.0	90	0.33
Cheese II ("Tilci")	0.6	5.3	7.1	17	0.35

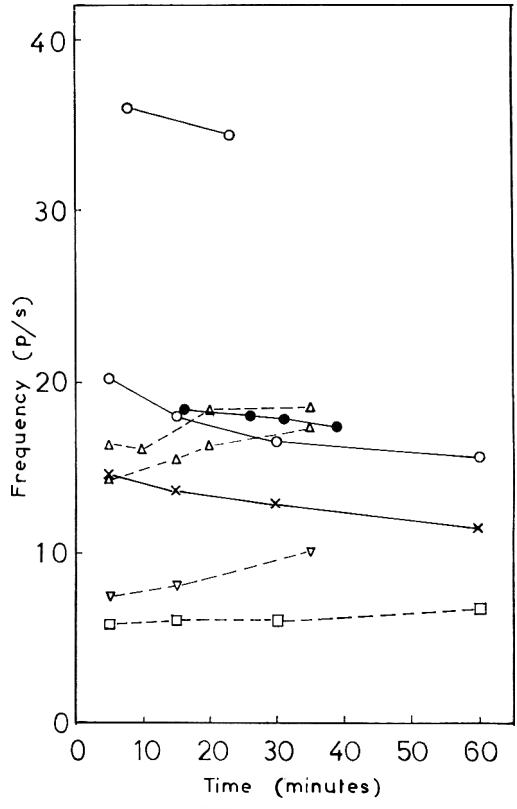


Fig. 9. Observed resonance frequency as a function of the time after cutting. ▽ = "Tilci" cheese; △ = "Herrgård" cheese; □ = fish pudding; ● = pear; x = potato; ○ = apple.

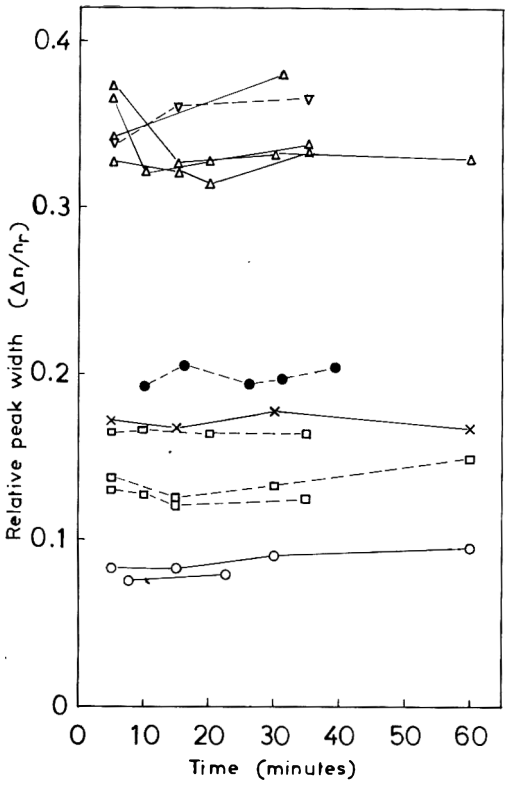


Fig. 10. Relative peak width $\Delta n/n_r$ as a function of the time after cutting (for explanations, see Fig. 9).

herein). However, with tissues that may become damaged by cutting, e.g., a fresh apple, evaporation control cannot be expected to prevent changes of the mechanical properties.

DISCUSSION

One of the purposes of this work is to give food technologists a better idea about the vibration properties of foodstuffs, and for that reason the method has been chosen so as to be elucidative rather than to afford a solution in terms of complex modulus, etc.

The apparatus described above should be considered as a device under development, and the results for cantilever bending are given only as an indication of the possibilities of the method.

As seen from Fig. 8, the peak for the pear is skew, whereas the peaks for the potato and for the cheese are almost as symmetrical as a theoretical curve. This indicates some non-linearity in the mechanical properties of the pear, a factor that in manual reading of isolated values could have been overlooked. Full discussion of such behavior, however, must be postponed to a later publication.

In comparison with manual methods of vibration analysis, it can be said that the automatic recording to a large degree reduces the personal factor, which may give interpersonal differences in readings, e.g., by adhering to different time schedules. Also, with the apparatus at hand, much more data can be collected during a workday; and, finally, a skewness or other irregularity of a curve can be detected more easily, when the whole resonance peak is visualized on a graph paper.

Further details of the apparatus and of the evaluation methods will be published in due course, together with more data on various foodstuffs.

ACKNOWLEDGMENT

The author thanks Professor E. von Sydow for making it possible to devote ample time to this project, Mr. K. Remi for skillful help with electronic constructions, and Dr. J. W. S. Hearle, Manchester, England, who in 1960 used an earlier model of the apparatus described here for measurements on textile fabrics, and with whom the author had valuable discussions.

REFERENCES

Anonymous. 1954. Objectivity comes to food research. *Chem. Eng. News.* **32**, 3356.

Bopp, C. D., W. K. Kirkland, O. Sisman, and R. L. Towns. Radiation effect on polymer vibration characteristics. 1947. *Ind. Eng. Chem.* **49**, 718.

Bradfield, G. 1960. Dynamic measurement of elasticity using resonance methods. *Brit. J. Appl. Phys.* **11**, 478.

Brooks, J., and H. P. Hale. 1959. The mechanical properties of the thick white of the hen's egg. *Biochim. et Biophys. Acta.* **32**, 237.

Grime, G., and J. E. Eaton. 1937. The determination of Young's modulus by flexural vibration. *Phil. Mag.* **23**, 96.

Halton, P., and G. W. Scott Blair. 1937. A study of some physical properties of flour doughs in relation to their bread-making qualities. *Cereal Chem.* **14**, 201.

Hartog, J. P. den. 1947. Mechanical vibrations. McGraw-Hill Book Co., New York, N. Y.

Kärrholm, E. M., and B. Schröder. 1953. Bending modulus of fibers measured with the resonance

frequency method. *Textile Research. J.* **23**, 207.

Kohlrausch, F. 1955. Praktische Physik. B. G. Teubner Verlagsges., Stuttgart, Vol. 1. Chap. 2.7. Mechanische Materialeigenschaften.

Moyal, J. E., and W. P. Fletcher. 1945. Free and forced vibration methods in the measurement of the dynamic properties of rubbers. *J. Sci. Instr.* **22**, 167.

Sack, H. S., J. Motz, H. L. Raub, and R. N. Work. 1947. Elastic losses in some high polymers as a function of frequency and temperature. *J. Appl. Phys.* **18**, 450.

Santen, G. W. van. 1953. Introduction to a study of mechanical vibration. Philips' Technical Library, N. V. Wed. J. Ahrend & Zn, Amsterdam.

Schuler, M. 1958. Mechanische Schwingungslehre, Teil I, Einfache Schwinger. Akad. Verlagsges., Leipzig.

Shimizu, T., and A. Ichiba. 1958. Rheological studies of wheat flour dough. I. Measurements of dynamic viscoelasticity. *Bull. Agr. Chem. Soc. Japan.* **22**, 294.

Virgin, H. I. 1955. A new method for the determination of the turgor of plant tissues. *Physiol. Plantarum.* **8**, 954.

Complexity of Flavor and Blending of Wines

V. L. SINGLETON AND C. S. OUGH
Dept. of Viticulture and Enology, University of California, Davis

(Manuscript received August 7, 1961)

SUMMARY

Thirty-four pairs of similar, commercially acceptable wines and a freshly prepared 50-50 blend of each pair were scored for quality by a small expert panel. The composite scores of the blends did not differ to even a low degree of significance from the score of the highest-scoring member of the pair. The scores of the blends were very highly significantly better than the scores for the low-scoring wines or the mean of the low- and high-scoring wine in each pair. In 7 cases among the 34 the blend's composite score was higher than that of the highest-scoring wine, and in no case was it lower than that of lowest-scoring wine of the pair. Increased complexity of flavor is believed to be a major factor in the favorable effect of blending shown in this study; other possible effects are discussed.

Complexity has long been considered a desirable factor in the quality of most flavorsome or odorous products. In cookery or perfumery, efforts are made to improve the product by adding many ingredients in amounts small enough to influence flavor or odor without being individually obvious. In wines, flavor complexity is considered very important to high quality and is believed to be one of the primary effects produced by proper aging. Although much blending of different lots of wine is done commercially, largely to improve or standardize the average final product quality, this blending remains largely an art. So far as the authors have been able to determine, there are no published studies on any product that demonstrate the value of a complex flavor or estimate the value of blending in producing increased complexity. Of course, correcting a frank deficiency or meeting uniformity standards by blending is an obviously similar problem and one relatively easily solved, but can blending or flavor modification within the limits of a single commercially acceptable product be expected to improve it, and, if so, how much?

As a part of a research program upon the aging of wines, experimental and less subjective evidence was sought testing the validity and estimating the magnitude of the presumed favorable effect of flavor complexity on wine quality.

EXPERIMENTAL METHODS

Pairs of wines were sought that were similar as to type, scored similarly as to quality and within the commercially acceptable range, and yet appeared to have different flavor qualities. The wines from which selections were made were the 1960 vintage produced in the University winery by standard production methods. Most of the grapes were from the University vineyards at Davis or Oakville, and the wine lots were each from a single grape variety. The wines were presented to a small (10-member) panel whose members had had considerable experience as sensory panelists and were familiar with wines. The manner of presentation and scoring was essentially as reported by Ough and Baker (1961), except that 10 samples were tasted each day and these were grouped as to type (and usually vineyard region), e.g., dry red table wines one day, red dessert wines the next, dry white table wines the third, etc. The wines were first scored during November–January, 1960–61, about three months after vintage. The average scores and comments were used to choose from among a given daily set of ten wine lots, two that had the most similar scores but the most different comments. These two lots, plus a sample prepared by blending equal volumes of these two, were retasted about five months later as part of a continuing panel evaluation of the 1960 vintage (during April–June, 1961).

The usual chemical analyses for alcohol, total acid, etc., were completed on each wine shortly after the first tasting. The wine lots were from 5 to 50 gallons each. The dry white wines were held in glass, cork-stoppered containers throughout the experiment with the exception of wine No. 3 (Table 1) which was in a 25-gallon previously used oak barrel until it was bottled on February 2, 1961. The other wines were in previously used oak cooperage throughout the experiment with the exception of wine Nos. (Table 1) 38, 41, 42, 45, 47, 48, 49, 50, 51, 56, 57, 58, and 66, which were in glass, cork-stoppered containers,

Table 1. Origin and analyses of the selected wine pairs.

Wine no.	Grape variety [a]	Total acidity (g tartaric/ 100 ml)	Volatile acidity (g HOAc/ 100 ml)	pH	Extract (g solid/ 100 ml)	Sugar (g glucose/ 100 ml)	Ethanol (% v/v)	Tannin (g tannic acid/ 100 ml)	Color (arbitrary units)[b]	First tasting	Second tasting	
										Mean score	Comments	Mean score
Dry white table wines												
1	Sylvaner	0.76	0.027	3.11	2.6	0.1	12.8	0.02	7.0	12.7	rich, med. distinctive	15.4
2	Sylvaner	0.98	0.028	3.13	2.4	0.1	12.6	0.02	7.3	13.7	fresh, lo dist.	14.3
blend											fresh, full med. dist.	15.3
3	Pinot blanc	0.62	0.022	3.35	2.2	0.1	10.6	0.03	7.0	14.0	hi dist., slt. stemmy	14.0
4	French Colombard	1.03	0.022	2.93	2.9	0.2	10.3	0.02	7.2	13.6	very woody, tart, spicy	13.9
blend											hi dist., tart, slt. wood	15.8
5	Chenin blanc	0.73	0.016	3.35	2.4	0.1	12.0	0.02	5.6	13.2	fruity, young	14.6
6	Aligote	0.63	0.024	3.24	2.3	0.2	13.6	0.03	5.0	13.9	lo dist., slt. weedy, bitter	13.6
blend											fruity, lo dist.	14.3
7	Sauvignon blanc	0.80	0.020	3.12	2.8	0.1	13.7	0.02	6.9	15.8	med. dist., slt. fruity	16.0
8	Sauvignon blanc	0.81	0.013	3.17	2.9	0.1	12.4	0.02	3.6	15.6	med. dist., Sauv. blanc, tart	15.9
blend											med. dist., spicy, aromatic	15.9
9	White Riesling	0.70	0.016	3.19	2.5	0.2	13.0	0.03	7.7	14.2	weedy, overripe Riesling	15.1
10	Chenin blanc	0.85	0.019	3.13	2.6	0.1	10.9	0.02	3.3	13.9	acidulous	14.3
blend											fruity, distinctive, slt. oxid.	14.4
11	Aligote	0.58	0.032	3.33	2.4	0.3	13.3	0.02	2.6	16.6	med. dist., fruity, rich	16.4
12	Aligote	0.63	0.022	3.19	2.4	0.2	13.7	0.03	2.9	15.1	med. dist., clean, slt. thin	15.3
blend											med. dist., fruity, fragrant	15.8
13	Chenin blanc	0.66	0.012	3.32	2.4	0.1	10.9	0.02	2.6	14.2	med. dist., slt. oxidized	15.1
14	Clairette blanche	0.49	0.022	3.52	2.6	0.1	12.9	0.02	10.7	13.8	lo dist., slt. meaty, thin	14.1
blend											lo dist., clean, balanced	14.9
15	French Colombard	0.79	0.016	3.15	2.6	0.1	12.2	0.02	3.3	11.6	fruity, odd aftertaste	14.9
16	Sémillon	0.54	0.018	3.68	4.0	0.1	13.6	0.03	7.1	12.4	med. dist., full	14.1
blend											med. dist., clean, SO_2	14.8
17	White Riesling	0.76	0.018	3.19	3.3	0.6	13.7	0.03	3.4	16.7	hi dist., full	16.9
18	Emerald Riesling	0.87	0.025	2.90	2.8	0.1	13.2	0.03	3.8	15.7	hi dist., rich	15.6
blend											hi dist., fruity, fresh	17.3

Table 1. Origin and analyses of the selected wine pairs (cont'd).

Wine no.	Grape variety [a]	Total acidity (g tartaric/ 100 ml)	Volatile acidity (g HOAc/ 100 ml)	pH	Extract (g solid/ 100 ml)	Sugar (g glucose/ 100 ml)	Ethanol (% v/v)	Tannin (g tannic acid/ 100 ml)	Color (arbitrary units)[b]	First tasting	Second tasting	
										Mean score	Comments	Mean score
19	St. Emilion	0.54	0.018	3.58	2.8	0.3	13.5	0.03	16.7	14.8	vinous, soft	13.5
20	St. Emilion	0.58	0.018	3.71	3.0	0.3	14.3	0.03	4.6	14.5	vinous, thin, slt. flat	13.3
blend											vinous, slt. flat	13.5
21	St. Emilion	0.61	0.016	3.61	2.8	0.1	14.9	0.03	3.6	13.5	med. dist., clean	13.8
22	St. Emilion	0.65	0.015	3.34	2.7	0.1	11.9	0.03	4.0	13.1	vinous, slt. bitter	13.4
blend											vinous, fresh, balanced	14.6
23	St. Emilion	0.57	0.016	3.49	3.0	0.2	13.1	0.04	3.7	14.1	med. dist., slt. bitter	14.0
24	St. Emilion	0.54	0.028	3.54	3.1	0.6	12.6	0.03	3.3	14.5	med. dist., bitter	13.5
blend											med. dist., tart	13.8
25	St. Emilion	0.54	0.016	3.63	2.8	0.2	14.2	0.03	5.6	13.0	med. dist., full	13.1
26	St. Emilion	0.52	0.018	3.78	3.1	0.2	13.5	0.03	5.3	11.9	med. dist., slt. skunky	11.9
blend											med. dist., slt. bitter	13.0
27	Folle blanche	0.65	0.040	3.18	2.9	0.2	9.9	0.03	5.6	13.5	med. dist., tart	12.2
28	Flame Tokay	0.43	0.026	3.88	2.5	0.1	11.2	0.02	5.6	13.9	med. dist., fruity, slt. off	11.3
blend											med. dist., fruity, tart	13.3
29	French Colombard	0.84	0.023	3.00	2.9	0.2	12.5	0.03	7.7	15.1	lo dist., slt. bitter, tart	15.1
30	Furmint	0.65	0.018	3.78	3.2	0.2	12.9	0.03	4.6	14.0	med. dist., overripe grapes	13.7
blend											med. dist.	14.0
Dry red table wines												
31	Pinot noir	0.72	0.017	3.47	3.2	0.1	11.4	0.20	167	14.5	distinctive Pinot	14.6
32	Pinot Pernand	0.58	0.022	3.62	3.4	0.1	13.4	0.12	111	13.8	sla. oxidized, malolactic	11.8
blend											Pinot, malolactic, wood	13.8
33	Gamay Burgogne	0.72	0.031	3.32	3.2	0.1	10.5	0.05	31	13.3	med. dist., fruity, rose, wood	12.4
34	Valdepenas	0.71	0.022	3.79	3.4	0.1	11.9	0.14	117	14.7	lo dist., oxidized, med. red	11.9
blend											complex, wood	14.2
35	Meunier	0.72	0.017	3.66	3.3	0.2	13.5	0.15	110	13.4	wood, slt. flat, fruity	14.4
36	Gamay Beaujolais	0.71	0.017	3.67	3.1	0.3	14.4	0.14	100	13.1	oxidized, odd flavor	11.4
blend											wood, slt. Pinot, slt. flat	14.3

Table 1. Origin and analyses of the selected wine pairs (cont'd).

Wine no.	Grape variety [a]	Total acidity (g tartaric/ 100 ml)	Volatile acidity (g HOAc/ 100 ml)	pH	Extract (g solid/ 100 ml)	Sugar (g glucose/ 100 ml)	Ethanol (% v/v)	Tannin (g tannic acid/ 100 ml)	Color (arbitrary units) [b]	First tasting	Second tasting	
										Mean score	Comments	Mean score
37	Selection 37	0.70	0.019	3.49	3.3	0.2	12.6	0.15	200	14.7	wood, rich	15.6
38	Selection 545	0.86	0.025	3.58	3.5	0.3	13.8	0.35	500	14.9	fruity, slt. bitter	15.1
blend											slt. wood, rich tannic	15.6
39	Malbec	0.49	0.038	4.13	3.3	0.1	11.6	0.14	220	12.1	hi dist., some bouquet	15.8
40	Cabernet Sauvignon	0.40	0.050	3.26	3.4	0.1	10.0	0.09	312	12.0	med. dist., malolactic, horsey	12.3
blend											hi dist., malolactic, rich	15.0
41	Selection 682	1.06	0.020	3.08	3.4	0.9	13.4	0.27	500	15.0	berry, hard and rough	14.6
42	Selection 547	1.03	0.019	3.12	3.4	0.2	11.6	0.33	500	14.4	full, fruity, acid	14.0
blend											fruity, bitter, tannic	14.4
43	Ruby Cabernet	0.86	0.015	3.47	3.4	0.1	12.6	0.26	500	15.9	weedy, Ruby Cabernet	16.2
44	Cabernet Sauvignon	0.53	0.028	3.78	3.1	0.2	13.5	0.18	334	16.2	wood, Cabernet type	15.9
blend											hi dist. Cabernet, wood, rich	16.9
45	Tannat	0.93	0.025	3.61	3.8	0.1	13.3	0.33	500	14.9	tannic, young, med. dist.	15.6
46	Petit Verdot	0.73	0.033	3.88	3.1	0.2	13.2	0.21	333	14.6	wood, rich, malolactic	14.2
blend											fruity, young, harsh note	14.6
47	Selection M63-18	0.89	0.012	3.56	3.7	0.2	10.9	0.16	111	13.6	lo dist., fruity	13.5
48	Selection M60-29	0.72	0.018	3.71	3.4	0.1	12.9	0.14	167	14.5	lo dist., harsh	11.9
blend											med. dist., slt. bitter, slt. oxid.	12.6
49	Selection M59-21	0.58	0.036	3.84	3.4	0.1	11.6	0.17	111	13.9	bacterial, slt. off, slt. metallic	12.6
50	Selection M58-54	0.49	0.051	4.26	4.1	0.1	12.4	0.19	167	12.7	bacterial, bitter, slt. ropey	10.6
blend											slt. off, lactic sour	12.6
51	Ruby Cabernet	0.79	0.020	3.67	3.5	0.1	13.1	0.23	500	15.3	hi dist., Ruby Cabernet	16.0
52	Ruby Cabernet	0.79	0.017	3.52	3.1	0.1	12.4	0.63	500	15.6	fruity, wood	15.3
blend											med. dist. Cabernet	15.5
53	Zinfandel	0.75	0.017	3.65	3.6	0.1	14.4	0.18	250	14.6	vinous, bitter	14.0
54	Calzin	0.82	0.030	3.29	3.8	0.5	14.9	0.48	333	13.9	dessertish, tannic	12.4
blend											vinous, tannic, bitter	13.6

Table 1. Origin and analyses of the selected wine pairs (cont'd).

Wine no.	Grape variety [a]	Total acidity (g tartaric/ 100 ml)	Volatile acidity (g HOAc/ 100 ml)	pH	Extract (g solid/ 100 ml)	Sugar (g glucose/ 100 ml)	Ethanol (% v/v)	Tannin (g tannic acid/ 100 ml)	Color (arbitrary units)[b]	First tasting Mean score	Second tasting Comments	Second tasting Mean score
55	Barbera	0.95	0.023	3.22	3.4	0.2	13.3	0.17	500	13.8	med. dist., wood	15.7
56	Carignane	0.51	0.018	3.69	3.2	0.1	12.7	0.12	125	14.3	vinous, slt. harsh	13.5
blend											lo dist., wood	14.2
White sweet fortified wines												
57	Early Muscat	0.43	0.020	3.98	15.4	11.0	19-20	0.03	8.4	15.8	hi dist., muscat, hot, heavy	16.9
58	July Muscat	0.28	0.016	4.21	17.2	13.4	19-20	0.03	8.3	15.6	fruity, slt. off	14.6
blend											hi dist. muscat, slt. hot, heavy	16.2
59	Fernão Pires	0.39	0.021	4.00	17.6	12.0	19.3	0.03	5.3	14.9	dessert, fruity, slt. hot	14.3
60	Mission	0.37	0.032	3.58	16.4	9.7	19-20	0.05	3.3	14.6	lo dist., slt. hot	13.7
blend											dessert, slt. oxidized, slt. hot	14.6
Red sweet fortified wines												
61	Royalty	0.48	0.014	3.92	16.0	13.2	19.6	0.28	500	16.1	med. dist., unusual fruity	16.2
62	Tinta Madeira	0.64	0.018	3.65	14.6	10.3	19-20	0.09	142	15.4	prunish, med. dist.	15.7
blend											med. dist., leafy, smooth	16.0
63	Zinfandel	0.42	0.016	3.77	14.2	10.9	19-20	0.11	125	13.1	slt. fusel, slt. hot	14.2
64	Muscat Hamburg	0.44	0.012	3.99	14.2	7.9	19.3	0.06	40	14.0	fruity, smooth	13.5
blend											slt. muscat, med. dist.	14.2
65	Teraldico	0.50	0.014	4.27	11.5	8.3	19-20	0.13	250	15.1	med. dist., smooth	15.4
66	Tinta Madeira	0.44	0.014	3.95	9.0	6.4	20.7	0.11	125	14.9	med. dist., slt. hot	14.9
blend											lo dist., smooth	14.9
67	Royalty	0.61	0.014	3.79	8.4	6.0	19.3	0.33	500	15.5	med. dist., bitter, medicinal	15.3
68	Tinta Madeira	0.56	0.022	3.98	15.2	7.5	19.6	0.16	200	14.9	med. dist., hot, rich	14.3
blend											med. dist., young, hot	15.3

[a] All wines from Davis vineyard except wines 37, 38, 41, 42, 54, and 55, from Oakville vineyard; wines 51 and 52, from a Napa Valley vineyard; wines 56 and 66, from a Madera vineyard; and wine 67, from a Stockton vineyard.

[b] Duboscq, color increases as values increase.

and wine 52, which was in wood, but was bottled about two months before its second tasting.

The second tasting included as a daily set the same ten wines as before plus the blended sample. The blend was prepared immediately before presentation of the samples to the panel. It was coded and randomized as to sequence, as were the other samples, so that the panelists were not aware of any special treatment and rated it as one of a group of eleven similar but unrelated individual wines. The scores recorded by each taster for each wine were based on the scale: 20–17, wines with some superior characteristics and no marked defects; 16–13, standard wines; 12–9, commercially acceptable wines with noticeable defects; 8–5, below commercial acceptance; and 4–1, completely spoiled. A minimum of six panelists tasted each day for which any results are recorded here. The flavor comments were those selected as most valid or revealing from the two or three panelists who included comments on their score sheets most frequently.

RESULTS AND DISCUSSION

Table 1 lists the origin and composition of the wine pairs chosen, the mean scores received in both tastings, and comments from the second tasting. If one compares the minimum detectable differences ($p = 0.05$, triangular test) in wine published by Hinreiner *et al.* (1955) with the analyses of each pair of wines, it is found that the following probably detectable concentration differences exist: sugar, pairs 57–8, 59–60, 61–2, 63–4, 65–6, and 67–8; alcohol, none; tannin, pairs 37–8, 51–2, 53–4, 61–2, and 67–8; and acid, pairs 1–2, 3–4, 9–10, 13–14, 15–16, 27–8, 29–30, 37–8, 43–4, 45–6, 47–8, 55–6, 57–8, and 61–2. Based on laboratory experience, the color differences large enough to have had a probable effect on scoring were in pairs 13–14, 19–20, 33–4, and 63–4. Eliminating duplicates, this totals 22, and thus 12 pairs of wines have no flavor or color differences that can be considered detectable on the basis of the available analytical and sensory information. Examination of mean scores of the pairs that do show analytical differences large enough to be sensorily significant (second tasting, Table 1) shows, for sugar, that the wine with lower sugar received the highest score in two cases, and the reverse was true in four cases. Only sweet wines are involved, and the differences are large enough that the blend would also be detectably different from the wines in only one or perhaps two cases of the six.

Similarly with tannin, only red wines are involved, and in the three dry-wine pairs the lower tannin got the higher score, but in the two sweet-wine pairs the higher tannin scored higher. In only two cases is the difference large enough that the blend would be expected to be detectably different from either of the wines based on tannin alone. Comparison of the tannin content and scores of several pairs of wines having high tannin both with and without sufficient difference to be a factor in scoring of the pair shows several wines having high scores and yet high tannin. Thus it seems impossible to correlate quality judgment with tannin content within this series of wines.

Total acidity calculated as tartaric gives even less clear relationships with quality score. In nine cases the high-acid wine was scored higher, but in five cases the low-acid wine got the higher score, and in only three cases would one expect the blend to differ detectably in acid from either of the wines. Wines with unusually high or low acid content received equally high scores in many cases. With color, the relation to quality score was exactly split among the four cases of sizable differences (one each favoring high and low color within the white wines and similarly within the reds). Several other color differences were probably large enough to be detectable but were within the commercial range and did not appear to correlate with quality score.

Considering the facts just mentioned, the fact that the wines often scored differently in the two tastings, and that the estimated detectable differences are based upon immediate cross-comparison in a triangular test whereas the scores here were obtained by judging a single wine at a time within a standard scoring system, it seems correct to conclude that in nearly all cases the crucial factors in quality scoring are not explained by simple differences in taste related to usual analytical values available for wines. This is in line with the results Baker and Amerine (1953) reported; even an estimating equation including several analytical determinations did not predict quality score

reliably. The authors therefore conclude that the quality score of a blend, if it is higher than one would expect from the scores of wines entering the blend, cannot be attributed (at least within this series of wines) to the simple supplementation of deficiencies. Although the blend of two wines with objectionably high and low acidities, etc., would probably result in an improved wine, our efforts to minimize this type of effect—by selection of pairs of wines for blending, and comparison scoring on only commercially acceptable, carefully matched pairs—appear to have been successful.

The data in Table 1 are so arranged that the wine receiving the highest mean score in the second tasting was numbered odd, and the even-numbered wines were those scoring lower. It may be worth noting in passing that this same relative order within each pair prevailed in 22 cases and was reversed in 12 cases in the first tasting. This suggests that relative quality usually appeared early and was fairly consistent in these wines. Since about five months intervened between the two tastings of each pair of wines, considerable development of these wines did occur. The mean scores of 32 wines increased, 32 decreased, and 4 remained the same in the second tasting as compared to the first, with a mean decrease of 0.1 score unit in the second tasting. This suggests that the panelists were either scoring in the first tasting partly on the basis of potential quality or that the temporary faults of the young wines such as lack of clarity and yeastiness were given less severe score deductions than later-appearing permanent defects such as oxidized flavors.

Although individual panelists did occasionally score a blend lower than either constituent wine, in no case (Table 1) did a blend receive a lower mean score by the whole panel than the lowest-scoring wine of the pair. In seven cases the blend received a higher mean score than either wine. A statistical evaluation of these results, considering the mean scores only, with no breakdown by tasters, etc., showed (Table 2) that the mean scores of the blends did not differ from those of the highest-scoring of the pair of wines blended, even to the lowest level of significance. However, the blend's scores were very highly significantly better than the average of the scores of the two constituent wines, and very highly significantly better than that of the lowest-scoring of the pair.

An analysis of variance of the scores given individually by five panelists who tasted most regularly (31 of these 34 pairs

Table 2. Tests for significance of the difference between the composite score for the blend and the composite scores for the wines in the blend.

	Difference of scores		
	Blend from higher wine	Blend from lower wine	Blend from mean of pair
(Student's t test, paired variates, null hypothesis, 33 degrees of freedom)			
Average deviation, $\overline{D}$	−.124	+1.00	+.438
Sums of squares of sample deviation minus average deviation, $\Sigma(D-\overline{D})^2$	17.401	22.02	11.176
$S_D = \sqrt{\frac{(D-\overline{D})^2}{33}}$	.726	.817	.582
$S_{\overline{D}} = S_D$	.126	.142	.101
$\sqrt{34}$			
$t = \frac{\overline{D} - O}{S_{\overline{D}}}$	.981	7.03	4.32
Significance, p	zero	>.001	>.001

Mean Score: All samples 14.39, all blends 14.68, all higher of pairs 14.81, all lower of pairs 13.68, mean of pair means 14.24.

of wines) gave the results shown in Table 3. A highly significantly higher score was found for the blend than for the mean score of the two wines. Of note among the interactions is that treatment didn't interact with wines or tasters. The taster × wine interaction is normal, as are the taster and wine significant variances. These data agree with the previous conclusion that a 50-50 blend of wines chosen as these were receives a better quality score than might be expected from the scores of the two wines. Considering that the analysis of variance shows such a high degree of variability with respect to difference between individual wines, scores given by individual tasters, and the reaction of individual tasters to different wines, the high significance level for the treatment is especially noteworthy. These data, then, experimentally confirm the value of blending wines for improved quality. If the blending of two standard-quality wines seldom gives a product as bad as the poorer, usually gives one (at 50-50 blend level) as good as the better one, and sometimes gives one better than either, blending appears capable of useful, if still rather empirical, extension. Much blending, as now practiced commercially, appears to be based on firmer footing, and probably is of more value than previously suspected.

The reasons for the improvement of a blend over its constituents cannot be stated with certainty, because our understanding of the composition-quality interrelationships in wines is far from complete. At least in some cases, however, the evidence points to an increase in complexity as a major contributor to the quality increase. By this is implied the addition of flavors whose absence cannot be considered a serious deficiency but whose presence in the proper amount contributes favorably. A further implication is that a flavor that may be undesirable when recognizably strong, may be a contributor to complexity and therefore not undesirable if below the recognition threshold in the blend. This may help explain the data reported by Kramer and Ditman (1956), who showed that cantaloupes treated with an insecticide were scored significantly better than untreated cantaloupes.

Still another factor may be the fact that differences sensed as if on a unitized scale are actually a geometric rather than linear function of the stimulus (Stevens, 1961). It thus appears that a flavorsome constituent sensed as a given strength in one wine, may not seem much weaker when diluted one-half with a wine lacking this constituent. For example, a simple solution containing one aromatic ester compared with that of a different ester both near minimum detectable levels may appear less odorous, less complex, and less satisfactory as odorants than a blend of equal amounts of both solutions.

Table 3. Analysis of variance. Treatment is a comparison of mean score of two wines and the score of blend of both.

Source	df	ss	ms	F
Total	309	962.80		
Tasters	4	109.91	27.47	24.30 **
Treatment	1	9.85	9.85	8.71 **
Wines	30	360.47	12.01	10.62 **
Treatment × wines	30	38.69	1.28	1.13
Tasters × treatment	4	4.87	1.22	1.07
Tasters × wines	120	313.12	2.60	2.30 **
Error (residual)	120	135.74	1.13	

** Significant to 1% level.

ACKNOWLEDGMENT

This research was supported in part by the Wine Advisory Board. The technical assistance of Elizabeth Shapkin and Walter Winton is gratefully acknowledged.

REFERENCES

Baker, G. A., and M. A. Amerine. 1953. Organoleptic ratings of wines estimated from analytical data. *Food Research* **18**, 381.

Hinreiner, E., F. Filipello, H. W. Berg, and A. D. Webb. 1955. Evaluation of thresholds and minimum difference concentrations for various constituents of wines. IV. Detectable differences in wine. *Food Technol.* **9**, 489.

Kramer, A., and L. P. Ditman. 1956. A simplified variables taste panel method for detecting flavor changes in vegetables treated with pesticides. *Food Technol.* **10**, 155.

Ough, C. S., and G. A. Baker. 1961. Small panel sensory evaluations of wines by scoring. *Hilgardia* **30**, 587.

Stevens, S. S. 1961. To honor Fechner and repeal his law. *Science* **133**, 80.

Determination of Odor Difference Thresholds

H. STONE, C. S. OUGH, AND R. M. PANGBORN
Departments of Food Science and Technology and Viticulture and Enology
University of California, Davis

(Manuscript received October 14, 1961)

SUMMARY

An olfactometer described previously was modified and used to determine odor-difference thresholds for 48 subjects at 4 levels of 2-heptanone. The apparatus was found to give rapid and reproducible threshold measurements. Difference thresholds were found to follow the Weber law. A Weber fraction of 0.23 mg/min/deviate was determined for 2-heptanone. The absolute threshold was found to be 8.97×10^{-4} mg of 2-heptanone/L air.

Previous investigations (Mrak *et al.*, 1959; Ough and Stone, 1961) have shown that measurements of odor-difference thresholds with an olfactometer could be reported in terms of concentration of the test substance in the inspired air rather than molar concentration of the test substance in a water, glycerol, or oil solvent.

The present work was conducted to establish difference thresholds for 48 subjects for 4 concentrations of 2-heptanone as well as to determine the absolute threshold. The validity of the methodology was tested, and the difference thresholds were studied to determine whether they followed Weber's law.

APPARATUS AND METHOD

Equipment. The olfactometer previously described in detail (Ough and Stone, 1961) has undergone several alterations. To improve ease of sealing the joints, all connections from the manifold to the main air stream were converted to ball joints sealed with odorless, non-reactive grease (Dow Corning 200 Fluid, Dow Corning Corp., Midland, Michigan) and held with C-clamps. Three stainless-steel valves (Whitey Needle Valves, Van Dyke Valve and Fitting Co., Oakland, California) were installed, one each between the stopcock and the flow meter, to provide more precise metering of air flow through the diffusion bulb. A double-walled water bath improved temperature control of the test liquid. To control internal air temperature, a cooling and heating system was installed in the air line. The air was first precooled and then heated to the desired operating temperature on passing through the heating coil. The temperature was set manually by a rheostat controlling the heating element. In addition, an air conditioner was installed to control room temperature. The inspired-air temperature for this study was $24 \pm 1°C$ at $40 \pm 5\%$ relative humidity.

Calibration of equipment. Air flow rates were determined by methods specified for the meters. To determine the actual amounts of 2-heptanone delivered to the hood, a modification of the Iddles and Jackson (1934) and Iddles *et al.* (1939) method was employed. The odor-saturated air (at varying flow rates) was trapped in a chloroform–dry ice bath, warmed to a liquid, and removed with pentane. Since the pentane did not freeze at this temperature (*ca.* $-80°C$), it was added before the traps were removed, to prevent any large loss of the 2-heptanone as it returned to a liquid state. The solution was then transferred to a beaker with the 2,4-dinitrophenylhydrazine, mixed thoroughly, and placed in a hood to remove the pentane and permit crystallization. After 24 hr the precipitates were harvested in sintered-glass crucibles and dried to constant weight. Control samples were included with each test run. Calibration by this technique revealed that only 63% of the 2-heptanone could be recovered; therefore this value was used as a correction term. Further investigation revealed that the 2-heptanone reacted directly with this reagent to the extent of 63%. The results of this calibration (Table 1) indicated that the actual recovered amounts were equal to the calculated amounts. The calculated amounts were therefore used in the calculations. The 2-heptanone was redistilled in the laboratory before use, and the vapor pressure was determined at the operating temperature by the static method. Calculations of the amount of odor delivered were by a method reported previously (Ough and Stone, 1961).

Table 1. Comparison of calculated amounts of 2-heptanone with recovered amounts.

Calculated amount (mg/min)	Recovered amount			
	Mean values	Std. deviation	Range	No. of recoveries
0.738	0.72	.....	.....	1
1.04	1.06	.....	.....	1
1.46	1.26	±.23	.925–1.45	4
1.91	2.11	.....	2.06–2.17	2
2.39	2.24	±.26	1.88–2.50	4
2.84	2.59	±.34	2.10–2.84	4
3.40	3.48	±.29	3.03–3.65	4
3.78	4.07	±.31	3.70–4.33	4
4.43	4.61	±.21	4.31–4.74	4
4.89	4.76	±.27	4.41–5.06	4

Panel and design. The panel was a group of 36 men and 12 women, 18–55 years old, selected from departmental staffs. About one-third of the panel had had experience with this type of testing. Individual sensitivities were determined at four levels of 2-heptanone (1.16, 2.14, 3.14, and 4.14 mg/min). Three concentrations were tested on each side of each level: 0.94, 1.00, 1.10, 1.24, 1.34, and 1.50 mg/min for series 1; 1.60, 1.90, 2.00, 2.24, 2.40, and 2.70 mg/min for series 2; 2.40, 2.74, 2.94, 3.34, 3.49, and 3.83 mg/min for series 3; and 3.04, 3.59, 3.83, 4.39, 4.67, and 5.2 mg/min for series 4. To convert these values to mg/L air, it is only necessary to divide these flow rates by 3.68×10^2 L per min (the flow rate of the main air stream).

The method used was the constant-stimulus method of paired presentation, regarded by Guilford (1954) as one of the most accurate of the psychophysical methods. The test method was modified slightly by randomization of the reference sample as well as the test samples. This would tend to eliminate any operational errors, and also help to suppress any position and order effects by the subjects. Panel members were asked to choose the sample within each pair with the more intense odor of heptanone; "no difference" responses were not permitted.

Seven randomized pairs were presented to each subject each day for 4 days. All pairs were randomized in all four series in one set of blocks. Responses to the first pair, which served as orientation, were not recorded. The effect of presentation of a sample of low concentration following a sample of high concentration was investigated by repeating the entire study with the panel tested on a single randomized series at one session. A definite testing time was assigned to each panel member. To provide incentive, panel members were told that rewards would be presented to all those completing the study.

To eliminate conversation between subject and investigator, a signal system was used: A green light on a signal board directly in front of the odor hood told the subject when a sample was presented. The subject indicated the more intense sample by pressing a button that lighted a bulb in front of the investigator. Each sample was presented for 10 sec, followed by 10 sec of purified air, with 15-sec intervals between pairs.

Statistical calculations. The method of maximum likelihood, explained by Jones (1957), was used to evaluate the data. The equation for the regression line and the standard errors of estimate of the deviates were determined. The data were plotted on normal-probability paper, with the percentage of responses termed high being plotted against stimulus concentration. The χ^2 analysis was used to check for fit and homogeneity.

Since the reference points covered a relatively large range of odor concentration, the data were tested for agreement with Weber's Law. Using a method described by Jones (1957), proportions of the difference stimuli to the constant stimulus were plotted against percentage of the responses called high. Maximum-likelihood solutions were determined at the four levels. Sums of squares and cross-products from the separate regressions were pooled, and a common regression was obtained for all observations. The difference between the sum of the residual sums of squares for the separate regressions and the residual for the common regression is caused by the difference between the separate regressions. If this difference, as χ^2, is not significant, the regression coefficients may be considered equal and estimated by the common regression coefficient. The remaining sum of squares may be partitioned into two components—coefficients and means. The χ^2 value of the coefficients should be insignificant if the data agree with Weber's Law. The other portioned parts will be

insignificant also if the data agree with the normal-response law.

To complete the study the absolute threshold for 2-heptanone was determined by the method of constant stimulus with air as a reference. Absolute threshold was defined as that concentration detected by 50% of the panel. Seven pairs were presented to each subject, with the first pair used for practice. Judges were informed that some of the concentration of odor would be very low, possibly below threshold, but that a "no decision" would not be allowed. A method outlined by Jones (1957) was used to determine the threshold value.

EXPERIMENTAL RESULTS

To determine the effect of training during the first study, the first day's samples were repeated on the fifth day and the data subjected to χ^2 analysis. The results are summarized in Table 2. Though the analysis showed no significant difference between the subjects from the first day to the fifth, some training had occurred as evidenced by the high χ^2 value of 7.46 (compared to the low of 1.48 for the second series). Therefore it was decided to delete the first day's data as representing training and familiarization with the apparatus.

Since the study covered a wide range of odor concentrations, order effects were tested. The data were analyzed by determining the number of correct responses of a sample pair in the 1.16-mg/min series when it followed itself or one of the higher-sample pairs (2.14, 3.14, or 4.14 mg/min). A test for homogeneity was carried out by χ^2 analysis. The results indicated that there was no significant heterogeneity; however, a definite trend was evidenced by the high value ($\chi^2 = 7.37$). Closer observation of the data by χ^2 analysis of the 1.16-mg/min series vs. the 2.14-mg/min series indicated an order effect of $\chi^2 = 5.8$ (significant at 5% probability level); therefore, the entire series of 4 levels of 2-heptanone was repeated. Instead of randomizing all levels, each series was randomized and tested separately, i.e., on day 1, the 6 pairs of the 2.14-mg/min series, and on day 2 the 3.14-mg/min series. The data were then subjected to χ^2 analysis for homogeneity compared with the completely randomized series. No significant difference was observed between the randomized series and the individual series ($\chi^2 = 0.42$); therefore the two series were combined and the difference

Table 2. Number of correct and incorrect responses on a daily basis for 48 subjects.

Test	Day	No. correct	No. incorrect	Total Pairs
A [a]	1	175	113	288
	2	190	98	288
	3	203	85	288
	4	199	89	288
Total		767	385	1152
B [b]	2	190	98	288
	3	203	85	288
	4	199	89	288
	5	194	94	288
Total		786	366	1152

[a] $\chi^2 = 7.46$ (not significant at 5% level).
[b] $\chi^2 = 1.48$ (not significant at 5% level)

Table 3. Maximum-likelihood solution at four reference points.

Constant-stimulus concentration	Source of χ^2	Analyses of regression df	Sum of squares	χ^2	Probability
A) 1.16 mg/min					
	Regression	1	1.3236	127.065	
	Residual	4	0.987	9.475	.05> p>.10
	Total	5	1.4223		
B) 2.14 mg/min					
	Regression	1	.8165	78.38	
	Residual	4	.1394	13.38	.01> p>.001
	Total	5	.9559		
C) 3.14 mg/min					
	Regression	1	1.259	120.86	
	Residual	4	.015	1.44	.80> p>.90
	Total	5	1.274		
D) 4.14 mg/min					
	Regression	1	1.4869	142.47	
	Residual	4	.050	4.8	.50> p>.30
	Total	5	1.5369		

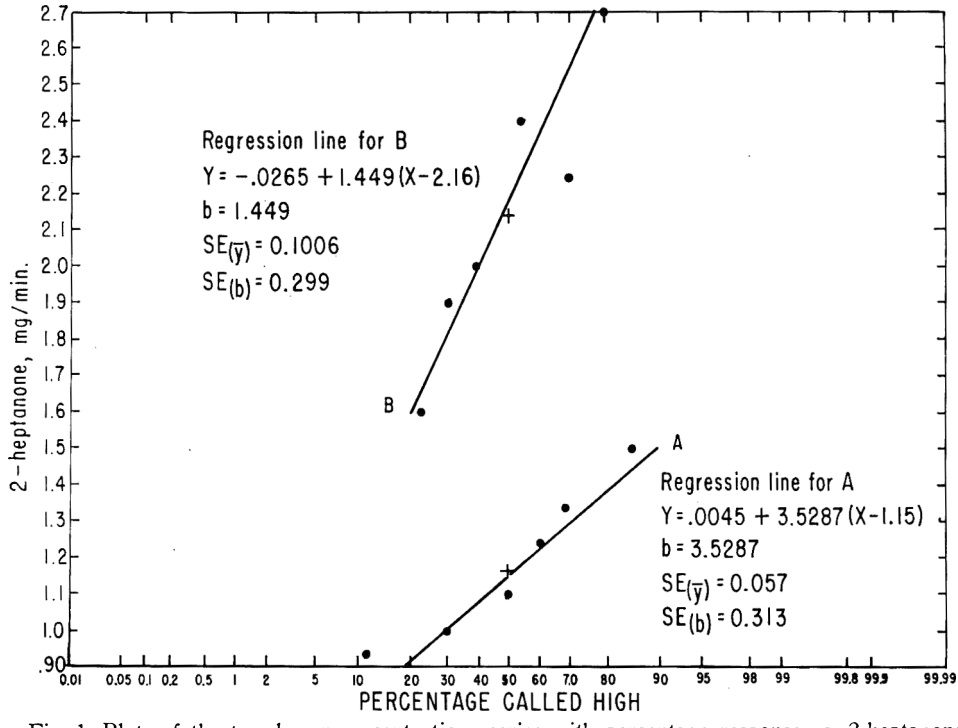


Fig. 1. Plots of the two lower-concentrations series with percentage response vs. 2-heptanone concentration and calculated regression lines and the standard errors of estimate.

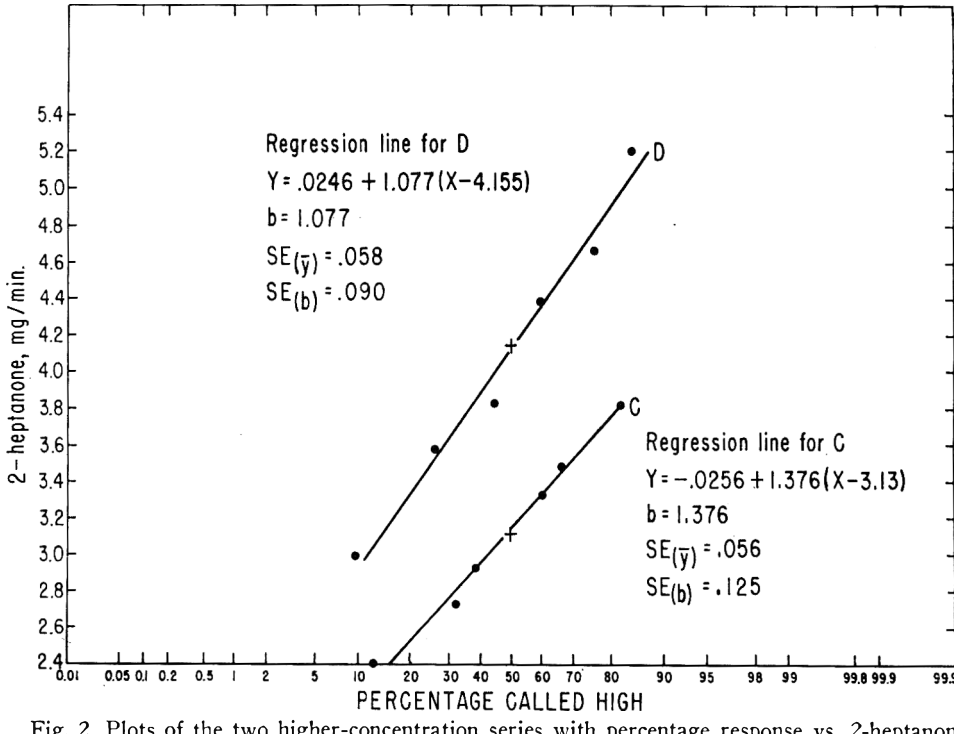


Fig. 2. Plots of the two higher-concentration series with percentage response vs. 2-heptanone concentration and calculated regression lines and the standard errors of estimate.

Table 4. Weber proportions, percentage response, Weber fractions, and residual χ^2 at each of four levels of 2-heptanone.

Reference level (mg/min) X_c							
1.16		2.14		3.14		4.14	
Weber [a] proportion $\frac{X}{X_c}$	Response called high (%)	Weber proportion $\frac{X}{X_c}$	Response called high (%)	Weber proportion $\frac{X}{X_c}$	Response called high (%)	Weber proportion $\frac{X}{X_c}$	Response called high (%)
.293	85.4	.262	79.2	.220	82.3	.256	84.4
.155	68.8	.121	54.2	.111	66.7	.128	76.0
.069	61.5	.047	69.8	.064	60.4	.060	59.4
−.052	50.0	−.065	39.6	−.064	38.5	−.075	44.8
−.138	30.2	−.112	30.2	−.127	32.3	−.133	26.1
−.190	11.5	−.252	22.9	−.236	12.5	−.266	9.4
Weber fraction $\frac{1}{b}$							
0.246		0.325		0.231		0.225	
χ^2 of residual (df = 4)							
10.19[b]		14.08[c]		1.32		4.82	

[a] X is the difference from the reference concentration (X_c).
[b] .05> p>.02.
[c] .0.1> p>.001.

thresholds were calculated. It was felt that the combined data would provide a more accurate measure of the difference thresholds for the 2-heptanone.

The percentage of responses called high vs. the 2-heptanone concentration (mg/min) was plotted on normal-probability paper (Figs. 1 and 2). The method of maximum likelihood was used for analysis. For each series, regression lines, slopes, and standard errors of estimate were calculated (Table 3). In only 1 series (2.14 mg/min) the regression analysis resulted in a significant residual term; therefore the standard error of estimate was based on the sum of squares of the χ^2 term. The regression coefficients in this study indicated, as would be expected, a greater sensitivity at the lower concentration [3.53 deviates/mg/min (A series)] than at the higher concentrations [1.449 deviates/mg/min (B series), 1.376 deviates/mg/min (C series), and 1.077 deviates/mg/min (D series)].

Test of Weber's Law. Table 4 gives the necessary transformed data for the maximum-likelihood calculations and the separate reciprocals of the regression coefficient and residual χ^2 value at each level. These Weber fractions are very similar except for the 2.14-mg/min level. The calculations showed that a highly significant χ^2 residual was associated with this regression coefficient. (This was caused by the control values on the flow meter, which had poor control characteristics in this area, making it difficult to set the flows properly at these set points). The inclusion of the 2.14-mg/min level caused an increase in residual terms sufficient to invalidate the test (both Weber's Law and normal-response law). For the remaining 3 series, there were no significant residual χ^2 terms (Table 5), indicating close agreement with Weber's Law; a Weber fraction of 0.234 was found for 2-heptanone at the concentration levels studied. Also, no significant deviation from the normal-response law was found if the 2.14-mg/min series was removed from the calculations.

Threshold determination. Threshold determinations are summarized in Table 6. The study was conducted on 2 successive days, with 6 pairs tested each day. Four higher sample levels were deleted from the calculations since the range in the lower 8 values was sufficient for the determination. The

Table 5. Test of Weber relationship at three reference levels (analysis of regression).

Source of χ^2	df	SS_R	χ^2	Probability
Separate regressions	12	0.1701	16.33	.20 > p > .10
Means	2	0.0054	0.52	.80 > p > .70
Coefficients	2	0.0137	1.31	.70 > p > .50
Common regression [N = 96]	16	0.1892	18.16	.50 > p > .30

Weber fraction [a] $= \frac{1}{b} = 0.234$ mg/min/deviate

[a] Estimated by the common regression coefficient.

Table 6. Maximum likelihood solution for threshold of 2-heptanone.

Source of χ^2	df	SS	χ^2	Probability
Regression	1	1.5251		
Residual	6	0.1385	6.65	$.50 > p > .30$
Total	7	1.6636		

$Y = -.1120 + 8.1078\ (X - 0.3039)$
$M_d = 0.33$ mg/min
$SE_{(Md)} = 0.04$

48 subjects had a mean threshold of 8.97×10^{-1} mg/L air for 2-heptanone at 24°C.

DISCUSSION

This study has provided additional information on the use of this olfactometer for measuring odor thresholds. The problems associated with training the panel were minor, as evidenced by the χ^2 analysis for homogeneity of the data for days 1 to 4 vs. days 2 to 5. The constant-stimulus method, when analyzed by the maximum-likelihood solution, provided sufficient data to determine the difference thresholds for 2-heptanone at four concentrations, and to obtain the absolute threshold all within 11 days of testing.

When the data were tested by Weber's Law, there was good agreement over the range of concentrations studied. One of the errors that can occur in all testing methods reflects the learning effect. By use of constant-stimuli methods, learning effects are kept to a minimum. Any magnitude estimation scale or rating scale is certainly subject to gross errors of this type since the subject must first create a frame of reference and a scale in his mind. Ough and Baker (1961) reported that a period of learning was necessary before the scores become meaningful. The frame of reference is the constant stimulus in the constant method; no scaling system is used. If the "adaptive level" theory of Helson (1947) is to be accepted then it would be expected that the effect of the adaptive level on the constant method would be small. Briefly, the adaptive-level theory considers the effects of memory or past learning during the experiment, and effects of asymmetrical experimental designs. In the constant method, where learning effects are kept to a minimum and the designs of the experiments are reasonably symmetrical, the adaption level should be very close to the constant stimulus and have little effect on the apparent constant-stimulus concentration. With the present data, order effects were not evident in the randomized series. When a sample pair from series D (4.14-mg/min series) was followed by a sample pair from series A (1.16-mg/min series), subjects had no difficulty in their perception of the latter pair.

Plotted on normal-probability paper the data followed the normal-response law. Subjects gave a slightly greater percent correct response to sample pairs on the lower side of each series. This resulted in a slight skewness that was not great enough to affect the calculations.

REFERENCES

Guilford, J. P. 1954. "Psychometric Methods." 2nd ed., p. 597. McGraw-Hill Book Co., New York.

Helson, H. 1947. Adaptation-level as frame of reference for prediction of psychophysical data. *Am. J. Psychol.* **60**, 1.

Iddles, H. A., and C. E. Jackson. 1934. Determination of carbonyl compounds by means of 2,4-dinitrophenylhydrazine. *Ind. Eng. Chem., Anal. Ed.* **6**, 454.

Iddles, H. A., A. W. Low, B. D. Rosen, and R. T. Hart. 1939. Determination of carbonyl compounds by means of 2,4-dinitrophenyl hydrazine. Water-insoluble carbonyl compounds. *Ind. Eng. Chem., Anal. Ed.* **11**, 102.

Jones, L. V. 1957. Methodology of preference measurements. Report p-1101, No. 4. Quartermaster Food and Container Inst. for the Armed Forces. 202 p.

Mrak, V., M. A. Amerine, C. S. Ough, and G. A. Baker. 1959. Odor difference test with applications to consumer preferences. *Food Research* **24**, 574.

Ough, C. S., and G. A. Baker. 1961. Small panel sensory evaluations of wines by scoring. *Hilgardia* **30**, 587.

Ough, C. S., and H. Stone. 1961. An olfactometer for rapid and critical odor measurements. *J. Food Sci.* **26**, 452.

All-Vegetable Protein Mixtures for Human Feeding VIII. Biological Testing of INCAP Vegetable Mixture Nine in Rats[a,b]

R. BRESSANI, L. G. ELIAS, AND N. S. SCRIMSHAW[c]
Institute of Nutrition of Central America and Panama (INCAP), Guatemala, C. A.

(Manuscript received December 15, 1961)

SUMMARY

The nutritive value of INCAP Vegetable Mixture 9 in young and protein-depleted adult rats was studied. This mixture contains 28% lime-treated corn, 28% sorghum grain, 38% cottonseed flour, 3% kikuyu leaf meal, and 3% torula yeast. When Mixture 9, skim milk, casein, or meat flour was fed combined with different amounts of starch to give a range of dietary protein levels, the nutritive value of the vegetable mixture for rats compared favorably with that of animal proteins at the higher levels of protein intake. Both young growing rats and protein-depleted rats were used. The growth response was generally less to the vegetable mixture at low protein levels than when animal proteins were fed at the same dietary level, presumably because of a lysine deficiency also observed in previous chick trials.

When the corn and sorghum combination was replaced in the mixture by processed corn, sorghum, rice, whole wheat, or oats, no significant changes were noted in the nutritive value. Furthermore, roasting, boiling, and lime-treating corn and sorghum did not affect the nutritive value of the mixture.

INCAP Vegetable Mixture 9 contains 27.5% protein, of which approximately 70% comes from cottonseed flour, 25% from corn-sorghum mixtures, and 5% from yeast protein (Bressani *et al.*, 1961b). Extensive studies in chicks (Bressani *et al.*, 1961a) have shown it to be free of toxic effects and relatively high in protein quality. Its nutritive value has also been confirmed by nitrogen-balance studies in children and by its use as the sole source of protein in the treatment of kwashiorkor (Scrimshaw *et al.*, 1961). Additional information on the nutritive value of INCAP Vegetable Mixture 9 was obtained from experiments with young and adult rats before the clinical studies were undertaken. This paper gives these results and compares Vegetable Mixture 9 with several animal proteins at various dietary levels of protein. It also describes the effects of various methods of processing the cereal grain employed in the mixture.

MATERIALS AND METHODS

Boiled corn and sorghum, grown and cultivated in the highlands of Guatemala, were prepared by cooking 1,000 g each of the two grains in the autoclave with 1,000 ml of water for 15 min, at 15 pounds pressure (131°C), and drying with hot air for 16 hr at 70°C. Germinated corn and sorghum were prepared by soaking 1,000 g each of corn and sorghum for 18 hr, germinating the seed between filter papers for 24 hr, and drying as with the boiled corn and sorghum. Roasted corn and sorghum were prepared by roasting equal weights of the two grains for 20 min in a small, electrically heated, rotary coffee roaster, with roasting temperatures varying from 180 to 220°F.

All grain preparations, when cooled and dried, were ground in a Wiley mill to pass 30 mesh and stored in bottles at 4°C until used. The source and description of the cottonseed flour, lime-treated corn, sorghum grain, and other ingredients of INCAP Vegetable Mixture 9 have already been

[a] This investigation was supported by grants RF-NRC-1 from the National Research Council (U. S.) and A-981 from the National Institutes of Health.

[b] INCAP Publication I-227.

[c] Present address: Department of Nutrition, Food Science and Technology, Massachusetts Institute of Technology, Cambridge, Massachusetts.

given (Bressani *et al.*, 1961a, b; Scrimshaw *et al.*, 1957).

The animal proteins used were skim milk (furnished by UNICEF), casein (V.F., Nutritional Biochemical Corp., Cleveland, Ohio), and meat flour. The meat-flour protein was prepared in the laboratory from prime-quality fresh local beef by drying for 24 hr with hot air at 70°C, grinding in a meat grinder, and then extracting with petroleum ether until free of fat. After a second drying the meat was ground in a Wiley mill to pass 40 mesh and stored at 4°C. This material contained 13.55% nitrogen.

The experimental animals were young and adult rats of the Wistar strain of the INCAP colony, assigned randomly by weight to the different experimental groups, so that the average initial weight per group was the same. Unless otherwise specified, 3 female and 3 male rats were used per group. They were placed in individual wire-screen cages with raised screen bottoms. Water and feed were given *ad libitum*. The young rats were weighed every 7 days during a 28-day growing period.

Adult rats were protein-depleted by feeding on a protein-free diet of 91% cornstarch, 4% cottonseed oil, 4% minerals (Hegsted *et al.*, 1941), 1% cod liver oil, and a complete vitamin solution previously described (Manna and Hauge, 1953). After losing 22–25% of their initial weight, they were fed the experimental diets for 14 days, with weight changes recorded each 7 days. Diet consumption was recorded and the diets were analyzed for nitrogen using the Kjeldahl method.

The base in all experiments was INCAP Vegetable Mixture 9 (Bressani *et al.*, 1961b), containing 28% corn, 28% sorghum, 38% cottonseed flour, 3% torula yeast, and 3% dehydrated leaf meal. Unless otherwise specified, the diets as fed contained 10% protein, obtained by diluting the 27.5% protein content of the mixture, and were supplemented with 5% cottonseed oil, 4% Hegsted mineral mixture, 1% cod liver oil, and a complete vitamin solution (Manna and Hauge, 1953).

RESULTS

Comparison with casein, skim milk, and meat flour. The data of Table 1 indicate the growth-promoting value of INCAP Vegetable Mixture 9 as compared to that of casein, skim milk, and meat fed at four different levels of protein in the diet. The diets were prepared by diluting the protein in the vegetable mixture (27.5% protein), in casein (89.5%), in skim milk (33.0%), and in meat flour (84.7%), to a calculated protein level of 5, 10, 15, 20, or 25%. Growth at all protein levels was slightly less for the vegetable mixture

Table 1. Effect of different corn-sorghum combinations on the nutritive value of Vegetable Mixture 9[a] (3 males, 3 females/group).

Variation in corn-sorghum combination[b]	Av. initial weight (g)	Av. weight gain (g)	F.E.[c]	P.E.R.[d]
I. Growth trial[e]				
100% corn	57	121	4.47	1.54
75% corn & 25% sorghum	57	112	4.70	1.46
50% corn & 50% sorghum	57	124	4.30	1.60
25% corn & 75% sorghum	57	120	4.53	1.52
100% sorghum	57	120	4.65	1.48
Casein & 0.3% cystine	59	103	3.27	1.91
II. Depletion-repletion trial[f]				
100% corn	131	63	4.21	1.63
75% corn & 25% sorghum	128	63	4.21	1.63
50% corn & 50% sorghum	139	60	4.45	1.54
25% corn & 75% sorghum	136	69	4.01	1.71
100% sorghum	136	70	3.95	1.74
Skim milk & 0.3% cystine	155	66	3.21	1.94

[a] Vegetable Mixture 9: 28% lime-treated corn, 28% sorghum, 38% cottonseed, 3% kikuyu leaf meal, and 3% torula yeast.

[b] All diets contained approximately 14.55% protein and were supplemented with: 5% NBCo No. 2 mineral mixture, 2% cod liver oil, 5% cottonseed oil, 2% alphacel, cornstarch to adjust to 100%, and 4 ml/100 g of a vitamin solution (Manna and Hauge, 1953).

[c] Feed efficiency: food consumed/weight gain.

[d] Protein efficiency ratio: weight gain/protein consumed.

[e] Experimental period lasted 28 days.

[f] Experimental period lasted 14 days.

than for casein. Feed efficiencies were similar. Protein efficiency ratios were higher with casein at low protein levels, but similar at the 10 to 25% levels for the two protein sources. With both Vegetable Mixture 9 and skim milk, growth increased in direct proportion to the increase in their protein levels, and feed efficiencies improved proportionately. Protein efficiency ratios were higher for skim milk than for casein or for Vegetable Mixture 9 at low protein levels, and similar at higher protein levels.

Only four protein levels were compared for Vegetable Mixture 9 and meat flour. Again, higher levels of protein in the diet resulted in higher weight gains from both protein sources, and at all protein levels the weight gains were the same for both the vegetable mixture and the meat flour. As protein levels in both foods rose, feed efficiencies also improved. Protein efficiencies were again slightly higher with meat flour than with Vegetable Mixture 9, particularly when the protein levels were low.

Effect of different corn-sorghum combinations. In these experiments, the corn and sorghum percentage distribution varied from 100% corn and 0% sorghum to 0% corn and 100% sorghum. The other ingredients of the mixture remained at 38% cottonseed flour, 3% torula yeast, and 3% dehydrated leaf meal, before adjustment at 10% protein. Table 2 gives the results of two trials. In the first, weight gains and feed and protein efficiencies were similar in both the 100% corn and 100% sorghum diets to those of the other groups. In the second, a protein-repletion experiment, the casein control group had less weight gain but the feed and protein efficiencies were similar to those of the other groups. The repletion weight gain and feed and protein efficiency for the skim milk control group were similar to those of any of the combinations of corn and sorghum in the basic formula, and none of these variations appeared to alter the protein value of the mixture.

Effect of treatment of the corn and sorghum. Because corn and sorghum are important in Latin-American diets (Bressani *et al.*, 1958, 1959; Bressani and Ríos, 1961), tests were made to determine the effect of various methods of preparation of these grains on the nutritive value of Vegetable Mixture 9. Five diets (partially described in Table 3), each containing approximately 15% protein, were fed for 28 days to 5 groups of rats. Weight gain was highest in the groups fed the vegetable mixture containing corn and sorghum, either raw, boiled in water, or cooked with lime. The other two groups fed the vegetable mixture with germinated or roasted corn and sorghum showed lower weight gains and feed protein efficiencies.

Substitution of other cereal grains. The same cereal grains or combinations, other than corn and sorghum, that were tested in chicks (Bressani *et al.*, 1959, 1961a) were also tested in rats. The experiment consisted of feeding variations of Vegetable Mixture 9 and a control diet of skim milk to 8 groups of rats. Table 3 gives the results and a partial description of the diets.

The oat cereal-based Vegetable Mixture 9 resulted in the best growth, slightly lower than that of the skim-milk control group. The descending order of weight gain for rats fed the other cereal grains was as follows: whole ground wheat, rice, wheat flour, and whole ground corn. The lime-treated corn-oat mixture and the lime-treated corn-rice mixture induced growth and feed efficiencies similar to that observed with the rice and wheat-flour diet, but lower than those of oats and higher than those for the mixtures containing only raw corn. For rats receiving either skim milk or any of the cereal diets, protein efficiencies were very similar except for the lower value for the group receiving wheat flour.

Comparative repletion of protein-depleted rats. Table 4 shows the results of two trials indicating that the vegetable mixture with 15% protein produced repletion weight gains comparable to those observed when the skim milk and casein or meat-flour diets were fed at the 10% protein level. Even at the 10% protein level the casein and vegetable mixtures gave similar repletion weight gains.

DISCUSSION

Previous (Bressani *et al.*, 1961a) and present testing of INCAP Vegetable Mixture 9 showed that the 56% of cereal in the corn and sorghum formula could be supplied equally by corn, sorghum, or any combination of the two. They also showed that ground rice, oat, and wheat or wheat flour may be substituted for the ground corn and sorghum in the basic formula without affecting nutritive value significantly.

The comparison of Vegetable Mixture 9 with various animal proteins further indicates that the mixture is of good nutritive value, particularly at higher protein levels. It is well known that amino acid deficiencies in proteins become more evident at lower levels of dietary protein than at higher levels, and an amino acid deficiency can often be corrected either by increasing the protein level in the diet or the protein intake (Harper, 1959). At levels lower than 10% of protein in the diet, the animal proteins induced better growth than the

Table 2. Twenty-eight-day comparisons between the nutritive value of Vegetable Mixture 9 and casein, skim milk and meat flour at different protein levels in the diet (3 male and 3 female rats/group).

Treatment	% test protein in diet	% protein content in diet	Av. initial weight (g)	Av. weight gain (g)	F.E.[a]	P.E.R.[b]
Vegetable Mixture 9 [c, d]	17.0	5.82	47	22	13.9	1.23
Vegetable Mixture 9 [c, d]	34.0	10.28	44	84	4.8	2.03
Vegetable Mixture 9 [c, d]	51.0	14.78	45	133	3.4	2.00
Vegetable Mixture 9 [c, d]	68.0	18.48	44	149	3.2	1.71
Vegetable Mixture 9 [c, d]	85.0	22.61	45	156	2.9	1.51
Casein [e]	5.0	5.73	47	30	9.5	1.83
Casein [e]	10.0	10.25	44	96	4.2	2.29
Casein [e]	15.0	15.31	45	142	3.0	2.14
Casein [e]	20.0	20.10	44	154	2.7	1.83
Casein [e]	25.0	24.91	44	184	2.3	1.73
Vegetable Mixture 9 [f]	18.5	5.16	50	17	17.6	1.14
Vegetable Mixture 9 [f]	37.0	10.58	50	89	4.6	2.04
Vegetable Mixture 9 [f]	55.6	15.48	50	125	3.5	1.85
Vegetable Mixture 9 [f]	74.1	19.60	50	129	3.1	1.67
Vegetable Mixture 9 [f]	90.0	24.50	50	147	2.9	1.42
Skim milk [g]	15.6	5.70	50	36	7.5	2.34
Skim milk [g]	31.2	10.26	50	117	3.4	2.88
Skim milk [g]	46.8	14.31	51	127	3.1	2.24
Skim milk [g]	62.4	19.62	50	148	2.6	1.94
Skim milk [g]	78.0	23.53	50	133	2.9	1.48
Vegetable Mixture 9 [h]	37.0	11.64	52	74	6.5	1.33
Vegetable Mixture 9 [h]	55.6	14.48	51	113	4.1	1.70
Vegetable Mixture 9 [h]	74.1	21.09	51	138	3.1	1.51
Vegetable Mixture 9 [h]	90.0	24.36	51	156	2.8	1.48
Meat flour [h]	11.1	11.21	51	54	6.0	1.49
Meat flour [h]	16.7	14.33	51	123	3.1	2.21
Meat flour [h]	22.3	19.81	51	130	3.2	1.60
Meat flour [h]	27.8	25.36	51	172	2.6	1.52

[a] Feed efficiency: food consumed/average weight gained.

[b] Protein efficiency ratio: average weight gain/average protein consumed.

[c] Lime-treated corn 28%, ground sorghum 28%, cottonseed flour 38%, kikuyu leaf meal 3%, and torula yeast 3%.

[d] All diets were supplemented with 4% NBCo No. 2 mineral mixture, 0.3% cod liver oil, (4.3, 3.6, 2.9, 1.4)% cottonseed oil, (1.9, 1.4, 1.0, 0.5)% alphacel, enough cornstarch to adjust to 100%, and 4 ml per 100 g of a vitamin supplement (Manna and Hauge, 1953).

[e] All diets were supplemented with 4% NBCo No. 2 mineral mixture, 0.3% cod liver oil, 5% cottonseed oil, 2.4% alphacel, enough cornstarch to adjust to 100%, and 4 ml per 100 g of a vitamin supplement (Manna and Hauge, 1953).

[f] All diets were supplemented with 4% Hegsted mineral mixture, 1.0% cod liver oil, 5% cottonseed oil, 10% cornstarch, sugar to adjust to 100%, and 4 ml of a complete vitamin supplement (Manna and Hauge, 1953).

[g] All diets were supplemented as in *f*, plus 2% celluflour.

[h] All diets were supplemented with 4% salmina mineral mixture (Bressani *et al.*, 1961b), 1% cod liver oil, 5% cottonseed oil, enough cornstarch to adjust to 100%, and 4 ml of a complete vitamin supplement (Manna and Hauge, 1953).

vegetable mixture, presumably because of amino acid deficiencies in the vegetable protein mixture. Previous studies showed that lysine in Vegetable Mixture 9 was limiting for the chick (Bressani *et al.*, 1961a), and the same appears to be true for rats when this mixture is fed at low protein levels.

The protein-efficiency ratio of Vegetable Mixture 9 at higher protein levels in the diet was similar to that of the animal proteins tested, suggesting that the nutritive value of the vegetable mixture is slightly lower than that of the animal proteins as tested in the young rat. The biological determination of nutritive value of Vegetable Mixture 9, however, showed it to be very similar to animal proteins in protein quality. Certainly it is possible to develop vegetable mixtures closely approaching the nutritive value of good-quality animal proteins such as eggs, milk, and meat. Such a conclusion is supported by the results obtained with the adult protein-depleted rats.

Since corn and sorghum are prepared in different ways for consumption in Central America, it was considered important to determine the extent to which the nutritive value of Vegetable Mixture 9 would be affected by the treatment. Most of the treatments tested are commercially available and

Table 3. Effect of the substitution of lime-treated corn and sorghum grain in Vegetable Mixture 9 by other corn and sorghum preparations and other cereal grains (3 male and 3 female rats/group, 28 days).

Variation in Vegetable Mixture 9 [a]	% protein in diet	Av. initial weight (g)	Av. weight gain (g)	F.E.[b]	P.E.R.[c]
Whole ground corn and sorghum grain [d,e]	13.50	61	132	3.5	2.10
Lime-treated corn and boiled sorghum [d,e]	14.10	61	140	3.5	2.04
Boiled corn and boiled sorghum [d,e]	14.20	61	128	3.5	2.02
Germinated corn and germinated sorghum [d,e]	14.40	61	120	3.8	1.81
Roasted corn and roasted sorghum [d,e]	13.70	61	126	3.7	1.99
Whole ground corn [f,g]	13.81	49	128	3.5	2.05
Whole ground wheat [f,g]	14.74	48	143	3.4	1.97
Oats [f,g]	15.58	50	151	3.3	1.93
White rice [f,g]	13.97	50	134	3.6	2.01
Wheat flour [f,g]	15.05	50	135	3.6	1.85
Lime-treated corn and oats [f,g]	15.07	49	133	3.4	1.95
Lime-treated corn and rice [f,g]	13.99	49	136	3.6	1.98
Skim milk [h]	16.28	49	158	3.0	2.07

[a] Vegetable Mixture 9: 28% lime-treated corn, 28% sorghum grain, 38% cottonseed flour, 3% kikuyu leaf meal, 3% torula yeast.

[b] Feed efficiency: food consumed/average weight gained.

[c] Protein efficiency ratio: average weight gain/average protein consumed.

[d] The percentages of cereal grains in *a* were replaced by the same percentage of the treated grains as indicated.

[e] All diets were supplemented with 1% cod liver oil, 4% Hegsted mineral mixture, 4% cottonseed oil, cornstarch to adjust to 100%, and 3 ml/100 g of a complete vitamin solution (Manna and Hauge, 1953).

[f] The percentages of cereal grains in *a* were replaced totally by the cereal indicated.

[g] All diets were supplemented with 1% cod liver oil, 4% Hegsted mineral mixture, 4% cottonseed oil, 20% sugar, 20% cornstarch and 3 ml/100 g of a complete vitamin solution (Manna and Hauge, 1953).

[h] This diet contained 46.8% skim milk, 2% celluflour, 21.2% sugar, 20% cornstarch, 1% cod liver oil, 4% Hegsted mineral mixture, 5% cottonseed oil, and 3 ml/100 g of a complete vitamin solution (Manna and Hauge, 1953).

Table 4. Repletion of protein depleted rats with Vegetable Mixture 9, skim milk, casein, whole egg, and meat flour.[a]

Treatment	% test protein in diet	% protein content in diet	Av. initial weight (g)	Av. weight gain (g)	F.E.[b]	P.E.R.[c]
Vegetable Mixture 9	37.0	10.19	158	48 [d]	5.6	1.74
Vegetable Mixture 9	55.0	15.03	158	59 [d]	4.2	1.58
Skim milk	31.3	10.48	158	71 [d]	3.5	2.72
Casein	11.0	9.70	158	69 [d]	3.8	2.70
Whole egg	15.5	10.04	158	76 [d]	3.3	3.03
Meat flour	12.0	10.60	158	75 [d]	3.5	2.69
Vegetable Mixture 9	37.0	10,58	175	41 [e]	3.2	3.00
Vegetable Mixture 9	55.0	14.09	174	55 [e]	2.4	2.91
Vegetable Mixture 9	74.0	19.29	175	66 [e]	2.0	2.57
Skim milk	31.3	10.05	175	51 [e]	2.3	4.30
Casein	11.0	8.77	175	42 [e]	2.9	3.96
Meat flour	12.0	9.94	175	52 [e]	2.6	3.93

[a] All diets were supplemented with 4% Hegsted mineral mixture, 5% cottonseed oil, 1% cod liver oil, cornstarch to adjust to 100%, and 4 ml of a complete vitamin solution (Manna and Hauge, 1953).
[b] Feed efficiency: food consumed/weight gain.
[c] Protein efficiency ratio: weight gain/protein consumed.
[d] 3 male and 3 female rats/group; experimental period, 14 days.
[e] 6 male rats/group; experimental period, 7 days.

could be used in the industrial production of vegetable mixtures.

Roasting and vapor-heating treatments have been shown most likely to decrease the already low nutritive quality of cereal proteins (Liener, 1950). Nevertheless, the experiments carried out with raw, lime-treated, boiled, roasted, or germinated corn and sorghum in the mixture showed that these treated cereals did not alter Mixture 9's nutritive value significantly. Since some of these treated cereals keep better than raw cereals, it would be advantageous to use them; the treatments destroy enzymatic activity, and the final product can thus be stored for longer periods.

The experiments also showed that a variety of other cereal grains can be substituted for corn or sorghum in the vegetable mixture without altering its nutritive value, providing the proportion of the protein from cottonseed flour is not altered. These make the Vegetable Mixture 9 formula more adaptable to parts of the world where corn and sorghum are not grown. Oats, whole wheat, and rice, actually improved the nutritive value of the mixture further, since corn, as is well known, has perhaps the lowest protein quantity among the cereals.

REFERENCES

Bressani, R., and B. Ríos. 1961. The chemical and essential amino acid composition of twenty-five selections of grain sorghum. *Cereal Chem.* (in press).

Bressani, R., R. Paz y Paz, and N. S. Scrimshaw, 1958. Corn nutrient losses. Chemical changes in corn during preparation of tortillas. *J. Agr. Food Chem.* **6**, 770.

Bressani, R., A. Aguirre, and N. S. Scrimshaw. 1959. All-vegetable protein mixtures for human feeding. II. The nutritive value of corn, sorghum, rice and buckwheat substituted for lime-treated corn in INCAP Vegetable Mixture Eight. *J. Nutrition* **69**. 351.

Bressani, R., A. Aguirre, L. G. Elías, R. Arroyave, R. Jarquín, and N. S. Scrimshaw. 1961a. All-vegetable protein mixtures for human feeding. IV. Biological testing of INCAP Vegetable Mixture Nine in chicks. *J. Nutrition* **74**, 209.

Bressani, R., L. G. Elías, A. Aguirre, and N. S. Scrimshaw. 1961b. All-vegetable protein mixtures for human feeding. III. The development of INCAP Vegetable Mixture Nine. *J. Nutrition* **74**, 201.

Harper, A. E. 1959. Sequence in which the amino acids of casein become limiting for the growth of the rat. *J. Nutrition* **67**. 109.

Hegsted, D. M., R. C. Mills, C. A. Elvehjem, and E. B. Hart. 1941. Choline in the nutrition of chicks. *J. Biol. Chem.* **138**. 459.

Liener, I. E. 1950. The effect of heat processing and storage on the nutritive value of proteins of importance in the cereal industry. *Trans. Am. Assoc. Cereal Chem.* **8**, 162.

Manna, L., and S. M. Hauge. 1953. A possible relationship of vitamin B_{13} to orotic acid. *J. Biol. Chem.* **202**, 91.

Scrimshaw, N. S., R. L. Squibb, R. Bressani, M. Béhar, F. Viteri, and G. Arroyave. 1957. Vegetable protein mixtures for the feeding of infants and young children. *In* "Amino Acid Malnutrition" (William H. Cole, ed.) p. 28. Rutgers University Press, New Brunswick, N. J.

Scrimshaw, N. S., M. Béhar, D. Wilson, F. Viteri, G. Arroyave, and R. Bressani. 1961. All-vegetable protein mixtures for human feeding. V. Clinical trials with INCAP Mixtures 8 and 9 and with corn and beans. *Am. J. Clin. Nutrition* **9**, 196.

Some Properties of the 2, 4-Dinitrophenylhydrazine Derivatives of Diacetyl, α-Acetolactic Acid, and Acetoin.[a]

During analyses of carbonyl compounds from the flavor concentrates of butter cultures, it was observed that, regardless of reaction conditions, α-acetolactic acid and acetoin yielded three derivatives with 2,4-dinitrophenylhydrazine (DNP): diacetyl bis-(DNP-hydrazone), diacetyl DNP-hydrazone, and acetoin DNP-hydrazone. Properties of the last two derivatives and methods for their separation have not been previously reported. This note relates methods for characterizing the above derivatives and reports some of their observed physical properties.

A mixture of the three aforementioned DNP-hydrazine derivatives resulting from α-acetolactic acid or acetoin may be separated into two fractions by the silicic acid procedure of Wolfrom and Arsenault (1960). The faster-moving fraction was identified as diacetyl bis-(DNP-hydrazone), whereas the slower of the two fractions was later found to contain a mixture of the DNP-hydrazones of acetoin and diacetyl. The slower-moving fraction, in ethylene chloride, was chromatographed on a column containing 1 part magnesium oxide (dried 380°C, 18 hr) and 10 parts Celite 545 (dried 150°C, 18 hr). An 18-mm-ID column containing 10 g of packing was adequate. Development with ethylene chloride gave two bands. The faster band, identified as acetoin DNP-hydrazone, appeared pink when the zone was compact on the column, and with diffusion it exhibited a tan color. The slower band, identified as the diacetyl DNP-hydrazone) gave a cherry red color on the column. Diacetyl bis-(DNP-hydrazone), if it were present, would appear as a blue band and would move slower than the mono derivative.

Properties of the authentic derivatives are given in Table 1. The distinguishing criterion for diacetyl DNP-hydrazone and acetoin DNP-hydrazone is the absorption maxima in chloroform and alcoholic KOH. It should be noted that the acetoin derivative differs from data reported previously (Patton *et al.*, 1958; Day *et al.*, 1960). A fraction identical to that reported by these workers was observed in the DNP-hydrazine derivatives of authentic acetoin, but infrared data did not reveal its identity.

Table 1. Properties of 2,4-dinitrophenylhydrazones.

2,4-DNP-hydrazone	Melting point (°C)	λ max chloroform	λ max alcoholic KOH	Color in alcoholic KOH
Bis-Diacetyl	318	430,394	540	Purple
Diacetyl	175	355	500	Reddish Pink
Acetoin[a]	178–79	327,259	510,382	Bright Pink

[a] Soluble in water.

Infrared spectra provide an additional method of differentiating DNP-hydrazones of acetoin and diacetyl. Spectra taken of the acetoin derivative in KBr pellets reveal strong absorption bands—a doublet, in the OH region at 3333 cm^{-1} and 3460 cm^{-1}—whereas the mono-DNP-hydrazone of diacetyl gave a strong band at 1685 cm^{-1}, characteristic of the carbonyl band.

It is evident that more suitable means should be employed for quantitative analysis of the aforementioned compounds, yet their DNP-hydrazine derivatives will occur during carbonyl analysis and must be accounted for. Gas chromatography provides a more suitable means of quantitative separation of diacetyl and acetoin ($Vg/Vg = 2.7$, acetoin: diacetyl, Apiezon M, 70°C). In this regard it was observed that acetoin issuing from the exhaust of a gas chromatograph had no odor.

R. C. Lindsay and E. A. Day
Department of Food and Dairy Technology

W. E. Sandine
Department of Microbiology
Oregon State University
Corvallis, Oregon

[a] Supported by funds granted by the American Dairy Association.

REFERENCES

Day, E. A., R. Bassette, and M. Keeney. 1960. Identification of volatile carbonyl compounds from Cheddar cheese. *J. Dairy Sci.* **43**, 463.

Patton, S., N. P. Wong, and D. A. Forss. 1958. Some volatile components of Cheddar cheese. *J. Dairy Sci.* **41**, 857.

Wolfrom, M. L., and G. P. Arsenault. 1960. Chromatographic separation of 2,4-dinitrophenylhydrazine derivatives of highly oxygenated carbonyl compounds. *Anal. Chem.* **32**, 693.