Journal of
FOOD SCIENCE

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Published by the Institute of Food Technologists six times a year. Printed by The Garrard Press, 510-524 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.

JULY-AUGUST, 1964 Volume 29, Number 4

An Official Publication of the Institute of Food Technologists

Journal of FOOD SCIENCE

JULY-AUGUST, 1964 Volume 29, Number 4

CONTENTS

Kinetics of Thermal Degradation of Chlorophyll in Spinach Puree [a]

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(Manuscript received September 20, 1963)

SUMMARY

The kinetics of chlorophyll degradation in spinach purée were studied over the temperature range 260–300°F, using thermal-death-time tubes heated in a constant-temperature oil bath. Pigments were separated by column chromatography, and their concentrations determined spectrophotometrically. A semilogarithmic (first-order) plot of the residual pigment vs. time was used to illustrate the thermal degradation rate (TDR). Although the major portions of the TDR curves were apparently linear, several abnormalities were observed during the initial test intervals. Regression coefficients were computed for the linear portions and used to compute the various thermodynamic functions characteristic of the degradation reactions. The following values respectively represent the average determinations for chlorophylls *a* and *b*. The TDR constant *k* (2.3/*D*, *D* being decimal reduction time in sec), 1.1 × 10^{-2} and 0.52 × 10^{-2} sec^{-1}; the temperature coefficient Q_{10} (log^{-1} 18/*z*, *z* being °F affecting 10-fold change in *k* or *D*), 1.58 and 1.26; the activation energy *E*, 143 and 35 Kcal $mole^{-1}$; the enthalpy *H*, 142 and 34 Kcal $mole^{-1}$; the free energy ΔF, 29 and 32 Kcal $mole^{-1}$; and the entropy ΔS, 268 and 5 cal deg^{-1} $mole^{-1}$.

INTRODUCTION

The degradation of naturally occurring colored pigments in food during processing is a major problem. In all green vegetables, the change in color from bright-green to dull olive-green or olive-yellow color is due to the conversion of chlorophylls to their respective pheophytins and further breakdown products such as pheophorbides and chlorins. This is a general degradation reaction occurring in all chlorophyllaceous foods. (Mackinney and Weast, 1940; Gold and Weckel, 1959; Westcott *et al.*, 1955).

With the advent of high-temperature short-time processing methods, it became possible to reduce the destruction of pigments in the processing step (Tan and Francis, 1962; Epstein, 1959). However, pigment degradation is rapid during storage, and the rate is independent of the previous equivalent processing dose (Epstein, 1959).

This study was made to determine the thermal-degradation kinetics of chlorophylls in spinach purée over a high temperature range and to examine some of the thermodynamic functions of the degradation reaction.

MATERIALS AND METHODS

Fresh spinach was washed and trimmed, and the leaves were blanched for 4 min in water at 175°F. The spinach was chilled immediately in cold water and drained. The blanched spinach was then puréed in a Fitzpatrick mill with a coarse screen and then a fine screen (No. 40). The puréed spinach was deaerated by allowing it to pass slowly from a separatory funnel into a desiccator maintained at a vacuum of 23 cm Hg. The deaerated purée was stored at 34°F until processed.

Conventional thermal-death-time (TDT) tubes were used to determine the kinetics of chlorophyll degradation at higher temperatures. TDT tubes prepared from Pyrex tubing (7 mm ID, 1 mm thickness, 18 cm length) were filled with purée using a glass tube of smaller diameter under air pressure. The open ends were sealed in an oxygen flame.

Tubes were heated in a temperature-controlled oil bath at 260, 270, 280, 290, and 300°F for 20, 40, 80, 160, and 320 seconds, followed by immediate cooling in ice water. After cooling, the tubes

[a] Contribution from the University of Massachusetts Agricultural Experiment Station.

were washed and stored at 0°F until analyzed for pigment content by the method of Tan and Francis (1962). Sixty tubes were used for each temperature-time condition, making a grand total of 1500.

RESULTS AND DISCUSSION

Thermal degradation rates (TDR) were determined at 260, 270, 280, 290, and 300°F from the regression coefficients at each temperature. Figs. 1 and 2 show the TDR curves for chlorophylls *a* and *b*, respectively. The solid lines represent the portions of the curves fitted by linear regression. It was observed that during the initial short heating period, pigment degradation was not first order, as evident from the dotted lines. The *D* values (*D* being the decimal reduction time in sec) were calculated from the slope of the TDR curves. Table 1 shows the *D* values obtained for chlorophylls *a* and *b* at various temperatures.

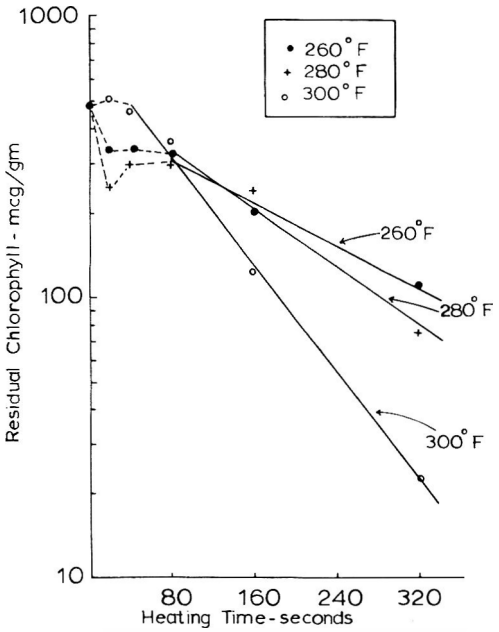

Fig. 1. Thermal degradation rates for chlorophyll *a* in spinach purée.

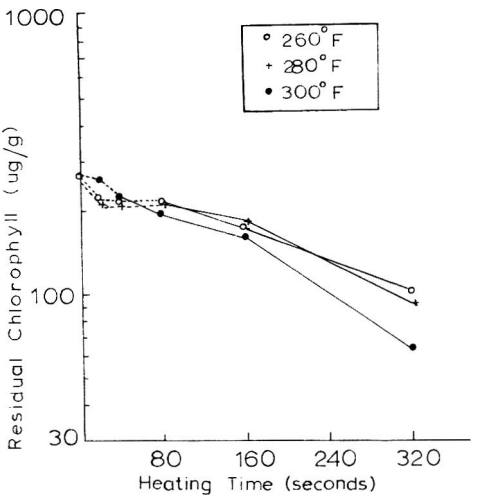

Fig. 2. Thermal degradation rates for chlorophyll *b* in spinach purée.

Table 1. Decimal reduction time (*D* values) for chlorophylls *a* and *b*.

Temp. (°F)	*D* values (sec)	
	Chlorophyll *a*	Chlorophyll *b*
260	584	756
270	452	667
280	350	588
290	271	518
300	210	457

D values were plotted against temperature on a semilog paper in order to obtain thermal reduction time (TRT) curves. The *z* values, computed from the slopes of the TRT curves, were 92 and 177°F for chlorophylls *a* and *b*, respectively. An Arrhenius plot of log *k* versus the reciprocal of the absolute temperature, where *k*, the degradation rate constant, = 2.3/*D* (Fig. 3), indi-

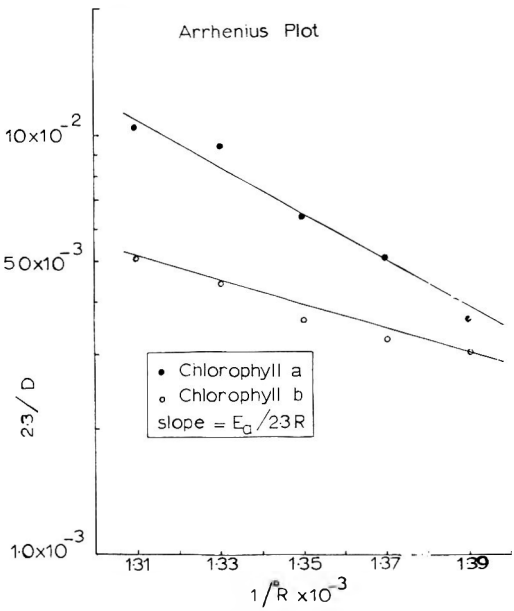

Fig. 3. Arrhenius plot for thermal degradation of chlorophylls *a* and *b* in spinach purée.

cated that the thermal degradation of chlorophyll *a* and *b* at 260–300°F followed typical first-order reaction kinetics. The energy of activation, *Ea*, for the degradation reaction, as calculated from the slope of the regression line (Table 2) showed values for this

Table 2. Activation energy and thermodynamic functions for thermal degradation of chlorophylls in spinach purée.

Function	Chlorophyll *a*	Chlorophyll *b*
pH of spinach purée	6.5	5.5
Temperature, °K	422	422
D, seconds (300°F)	210	457
$k = 2.3/D$, sec^{-1}	1.1×10^{-2}	5.2×10^{-3}
z value, °F	92	177
Q_{10}	1.58	1.26
E, Kcal/mole	143	35.2
ΔH, Kcal/mole	142	34.3
ΔF, Kcal/mole	29	32
ΔS, cal/deg mole	268	5.2

and other thermodynamic constants that were characteristic of thermal degradation reactions.

Energy of activation, *Ea*, was calculated from the slope of the Arrhenius plot. The slope of the regression line is $-Ea/2.3R$, where R is the gas constant. Temperature coefficients, Q_{10}, were calculated from the ratio of the degradation rate constants at two temperatures with 10°C difference or 18°F difference,

$$Q_{10} = \frac{k_t°\text{C}}{k_{(t-10)}°\text{C}} = \frac{k_t°\text{F}}{k_{(t-18)}°\text{F}}.$$

Knowing Q_{10}, it is possible to calculate the *z* value from the equation $Q_{10} = \log^{-1} 18/z$ (El-Bisi, 1955), where *z* is the °F effecting a 10-fold change in the degradation constant *k* or *D*. The enthalpy, ΔH, was calculated from the equation $\Delta H = E - RT$. The degradation rate constant, *k*, is exponentially related to free energy, enthalpy, and the entropy, by the following equations (Glasstone, 1960):

$$k = \frac{(K'T)}{h} e^{-\Delta F/RT} \text{ and } k = \frac{(K'T)}{h} e^{-\Delta S/R} \times e^{-\Delta H/RT}$$

where $K' = 1.380 \times 10^{-16}$ erg/deg. (Boltzmann constant)

$h = 6.24 \times 10^{-27}$ erg/sec. (Planck's constant).

ΔF, ΔS, and ΔH are respectively the free energy, entropy, and enthalpy of the reaction. T is the absolute temperature and R is the gas constant. The entropy of the reaction can be calculated from the equation $\Delta F = \Delta H - T\Delta S$. The higher energy of activation acquired by the chlorophyll *a* molecule and the higher entropy of the reaction suggests that chlorophyll *a* is a faster-reacting molecule, and hence thermally less stable, than chlorophyll *b*. This is also evident from the higher free energy possessed by chlorophyll *b* than *a*. It should be noted that these values are not the absolute values for chlorophyll *a* and *b*, because, in a pure system containing only the pigments, the values would be different.

Similar thermodynamic data have been calculated for proteins such as hemoglobin, trypsin, pancreatic lipase (Neurath *et al.*, 1944), and three species of bacteria, *Bacillus natto*, *Bacillus megatherium*, and *Bacillus myocoides* (Amaha, 1952). It is interesting to note that the spores of *Bacillus megatherium* are thermally more stable than the other two species. The Q_{10} values obtained are significantly higher in thermal death of bacterial endospores than in chemical reactions. Q_{10} values are also higher in the denaturation of proteins. The lower Q_{10} values for chlorophylls than for bacteria explains the higher residual pigment retention, and hence the color, in an HTST method than in a conventional thermoprocess of equivalent *F* value. The *F* value is defined as the time in minutes required for a given number of microorganisms or a given quantity of enzymes or other components to be reduced to a given level at a given temperature. A superscript should define the degree of destruction or reduction in terms of number of log cycles, while a subscript defines the temperature. In the canning of foods, the thermoprocess is usually computed to provide a 12-log cycle of destruction of *Clostridium botulinum* type *A* spores, or a process equivalent to F_{250}^{12}. In the case of enzymes, 3-log cycles of inactivation is usually considered sufficient. Fig. 4 shows the thermal-reduction-time curves for chlorophyll *a* and *b*, thiamine (Feliciotti and Esselen, 1957), and *Clostridium botulinum* (Kaplan *et al.*, 1954). Fig. 4 illustrates the

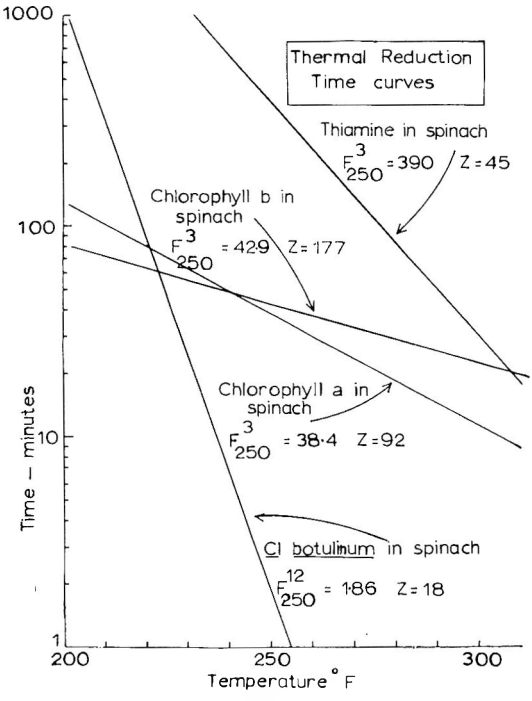

Fig. 4. Thermal destruction time curves for chlorophylls *a* and *b*, thiamine, and *Cl. botulinum* in spinach purée.

significant advantage in quality retention in terms of chlorophyll *a* and thiamine for an HTST thermoprocess when compared with a conventional process. It should be possible to calculate TRT curves for all thermolabile compounds and microorganisms in foods and arrive at an optimum compromise between nutrient retention, enzyme regeneration, and destruction of microorganisms. This concept is well known (Ball, 1957; Stumbo, 1963), and may become even more important when higher temperature ranges are introduced.

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Color Deterioration in Frozen French Beans (Phaseolus vulgaris)

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(Manuscript received July 1, 1963)

SUMMARY

Examination of 12 commercial frozen bean packs showed two forms of color deterioration. The first resulted in conversion of chlorophyll to pheophytin, and the second caused destruction of both chlorophyll and pheophytin. It is shown that both forms of degradation may be related to the process of fat peroxidation. The conversion of chlorophyll to pheophytin may occur during the anaerobic initiation stage of fat peroxidation. Destruction may occur during the aerobic propagation stage.

INTRODUCTION

The loss of natural color during the storage of frozen green vegetables has attracted much attention. Campbell (1937) showed that color deterioration in frozen peas stored above −18°C was due to conversion of chlorophylls *a* and *b* to the corresponding pheophytins. Mackinney and Weast (1940) and Dietrich *et al.* (1957) later proposed that the ratio of chlorophyll to pheophytin be used to measure color change in green vegetables.

It is well known that in acid solution the magnesium in chlorophylls is replaced by hydrogen to give the corresponding pheophytins, and Joslyn and Mackinney (1938) and Mackinney and Joslyn (1940) showed that the rate was first order with respect to acid concentration. This led Wagenknecht *et al.* (1952) to suggest that the accumulation of free fatty acids in stored frozen raw peas was responsible for their color loss, while Dietrich *et al.* (1957) suggested that the different acidities of peas and beans accounted for their different rates of chlorophyll conversion.

Other forms of chlorophyll degradation are also known. Chlorophylls are bleached during fat peroxidation (Strain, 1941) and during oxidation of glycolic acid by the enzyme α-hydroxyacid dehydrogenase (Kolesnikov, 1948, 1949; Tolbert and Burris, 1950). In addition, the enzyme chlorophyllase hydrolyzes the phytyl ester group of chlorophylls and pheophytins.

The present work demonstrates that two forms of chlorophyll degradation occur in stored frozen green beans. The first results in conversion of chlorophyll to pheophytin, and the second results in destruction of chlorophylls and pheophytins. A possible mechanism for the two forms of degradation is suggested.

MATERIALS AND METHODS

Preparation of pure chlorophylls. Chlorophylls *a* and *b* were prepared by sugar column chromatography of bean pigments by the method of Smith and Benitez (1955). The chlorophyll *a* and *b* zones were dug out separately and eluted with carbonyl-free ethanol. Spectrophotometric analysis showed that the *a* zone contained approximately 20% pheophytin *a* but less than 2% of other impurities and the only impurity in the *b* zone was 5% pheophytin *b*. These solutions were stable for at least 1 week when stored at −18°C in the dark.

Quantitative extraction of lipid. Lipid was extracted into chloroform by a slightly modified form of the method of Bligh and Dyer (1959). The modifications comprised the use of acidified methanol (0.04*N* HCl), in order to extract free fatty acids, and separation of the chloroform phase by centrifuging. Care was exercised to maintain low temperatures during extraction, by blending frozen beans with cold (−18°C) chloroform and methanol and centrifuging at 0°C.

Quantitative extraction of chlorophylls. The method used was similar to that for extracting lipids, but neutral methanol was used to avoid breakdown of the chlorophylls. Chlorophylls were stable for at least 9 days when stored at −18°C in the dark. Immediately prior to use 50 ml of

chloroform extract was evaporated to dryness (25°C and 8 mm Hg pressure) and the residues dissolved in 5 ml carbonyl-free ethanol.

Enzyme preparations. Soybean lipoxidase was obtained from Sigma Chemical Co., St. Louis, U.S.A. Bean enzymes were prepared by blending 5 g fresh beans with 45 ml water. The homogenate was centrifuged at 5,000 × G for 5 min and the supernatant solution used as the enzyme source.

Determination of lipoxidase activity. Enzyme activity was measured by the production of fat peroxide after incubating linoleic acid with a source of lipoxidase at 25°C. Linoleic acid was finely dispersed by adding 0.1 ml of a 0.25% w/v solution in ethanol to 2 ml of the aqueous reaction system which contained 0.2 mmoles sodium phosphate buffer (pH 7.0) and lipoxidase. After incubation, 5 ml ethanol, 0.2 ml of 1:1 hydrochloric acid, and 0.1 ml of 1% w/v ammonium ferrous sulfate solution were added to the 2 ml of assay solution. After a further 30 sec, 1 ml of 20% w/v ammonium thiocyanate was added and the absorbance at 480 mμ was read 3 min later.

Determination of chlorophylls and pheophytins. Chlorophylls and pheophytins were determined photometrically. The percentage conversion of chlorophyll to pheophytin was calculated by the method of Dietrich (1958). Concentrations of chlorophylls and pheophytins, expressed in μmoles/L, were calculated from the equations below, which were derived by matrix inversion of data from Aronoff (1953):

$$\text{Chlorophyll } a = -36.40D_{505} - 0.33D_{525} + 0.89D_{642.5} + 11.53D_{660}$$

$$\text{Chlorophyll } b = +1.70D_{505} - 0.83D_{525} + 19.37D_{642.5} - 3.11D_{660}$$

$$\text{Pheophytin } a = +110.38D_{505} - 53.51D_{525} - 2.46D_{642.5} - 0.67D_{660}$$

$$\text{Pheophytin } b = -41.15D_{505} + 112.49D_{525} - 3.96D_{642.5} - 0.79D_{660}$$

$$\text{Total} = 34.53D_{505} + 57.82D_{525} + 13.84D_{642.5} + 6.96D_{660}$$

Determination of free fatty acid. An aliquot (50 ml) of acid chloroform extract of beans was evaporated to dryness (8 mm Hg pressure, 25°C) and taken up in 25 ml of ethanol. Aliquots (10 ml) were titrated to pH 7.0 with 0.01*N* sodium hydroxide. When triplicate determinations were made on 5 replicate extractions of a batch of beans the standard deviation was 3%.

Determination of fat peroxide. Fat peroxide was determined in 1 ml of acid chloroform extract by the method used in the lipoxidase assay. When duplicate determinations of peroxide were made on the same 5 replicate extractions used to determine free fatty acids the standard deviation was 5%.

Determination of conjugated diene. Conjugated diene was determined from the absorbance of a sharp peak at 230 mμ of an acid chloroform extract diluted in ethanol. Concentration was calculated assuming a molecular extinction coefficient of 23,000.

Biochemicals. Linoleic acid (94–96% pure) was obtained from Fluka, Buchs, Switzerland.

RESULTS

Analysis of chlorophylls and lipids in commercial bean packs. Initial experiments were conducted on a series of commercial frozen bean samples produced in the 1961 and 1962 seasons. Examinations were made in October, 1962, when the samples were 3–22 months old. Fig. 1 shows

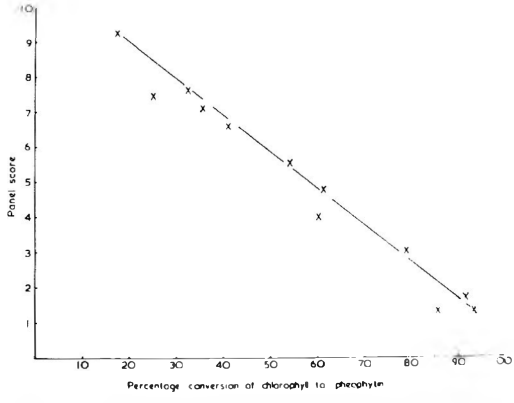

Fig. 1. The relationship of color appearance of green beans to % conversion of chlorophyll to pheophytin. Samples were assessed for color by a trained panel of 55. A score of 10 represents fresh beans, and 0 extreme yellowing.

that visual appearance of the beans was closely related to the extent of chlorophyll conversion to pheophytin. Table 1 shows that this conversion was progressive during storage. However, chlorophyll conversion to pheophytin was not the only reaction affecting color, since beyond 12 months' storage chlorophylls and pheophytins were progressively destroyed.

Table 1 also shows that the pH of beans remains relatively constant throughout frozen storage and the content of free fatty acids shows no trend with time, although fat peroxide increases. The increase of fat peroxide coincides with the start of chlorophyll and pheophytin destruction, but the concentration of diene shows no trend and is not related to the content of fat peroxide. However, many non-lipid compounds with a conjugated diene configuration are present in chloroform-methanol extracts and no such relationship would be expected.

Table 1. Analysis of twelve commercial bean packs, var. Tendergreen. The beans were obtained from commercial warehouses, at −18°C, and had been processed over the previous 22 months.

Months of storage at 0°F	Total chlorophyll + pheophytin (μmoles/100 g beans)	Conversion of chlorophyll to pheophytin (%)	Natural pH of bean homogenate	Free fatty acid (μeq/100 g beans)	Fat peroxide (μeq/100 g beans)	Conjugated diene (μmoles/100 g beans)
3	16.7	18.7	5.8	28	21.5	160.0
4	17.4	26.4	5.8	44	17.6	87.0
4	14.7	33.8	5.7	36	14.9	58.0
4	17.1	36.8	5.9	46	19.1	103.7
6	17.5	44.1	6.0	58	29.9	119.7
8	15.8	54.1	6.1	38	25.4	72.2
8	16.9	61.0	6.0	48	29.5	60.9
12	14.1	62.5	5.9	48	31.2	64.0
15	12.1	80.9	5.8	47	72.2	67.6
15	10.7	85.4	5.8	42	84.9	80.9
18	8.2	90.6	5.9	53	108.3	103.6
22	7.0	91.1	6.0	32	172.9	84.5

Table 2. Aerobic (I) and anaerobic (II) degradation of crude chlorophylls in the presence of linoleic acid and a homogenate of fresh beans.

A bean homogenate (0.1 ml) was used as a source of lipoxidase and the standard assay solution also contained 29 mμ moles crude chlorophyll. Aerobic incubation was in test tubes and anaerobic incubation was in Thunberg tubes evacuated and filled with nitrogen 5 times. After incubation chlorophylls were extracted with 3 ml of diethyl ether and determined.

Period of incubation (min)	Total chlorophyll + pheophytin (mμmoles)		Conversion of chlorophyll *a* to pheophytin *a* (%)		Conversion of chlorophyll *b* to pheophytin *b* (%)	
	I	II	I	II	I	II
0	29	29	15	12	0	0
2	30	29	38	47	10	15
5	29	30	87	93	27	32
10	21	29	100	100	63	70
20	14	28	100	100	91	87
30	7	29	100	100	95	96

The degradation of chlorophylls during fat peroxidation. Table 2 shows the effect of incubating linoleic acid with a homogenate of fresh beans and crude chlorophyll preparations. Chlorophylls became degraded to pheophytins, both aerobically and anaerobically, and chlorophyll *a* was degraded faster than chlorophyll *b*. After approximately 10 min of aerobic incubation, a further reaction began that led to destruction of chlorophylls and pheophytins. This second reaction did not proceed anaerobically.

When purified chlorophylls were used instead of the crude preparations, results were different. Table 3 shows that pure chlorophylls were not degraded to pheophytin when incubated anaerobically with linoleic acid and bean homogenate. Aerobically, chlorophylls were destroyed and there was no lag period.

Soybean lipoxidase (0.1 mg) had a similar effect to a bean homogenate on both crude and pure chlorophyll preparations.

Inhibition of fat peroxidation by chlorophyll preparations. The production of fat peroxide was measured when linoleic acid and soybean lipoxidase were incubated with either crude chlorophyll preparations or pure chlorophylls *a* and *b*. Table 4 shows that crude chlorophylls greatly inhibited fat peroxide formation for incubation periods up to 10 min, but on further incubation some peroxidation of fat occurred. Table 5 shows that the start of fat peroxidation coincided with the start of chlorophyll destruction.

To determine whether the lag period was due to the presence of an antioxidant or to accelerated breakdown of fat peroxide, the solutions were tested for malonaldehyde by the thiobarbituric acid test of Tarladgis (1963). Less than 3 mμmoles of malonaldehyde were formed during 30 min of incubation of crude chlorophylls with linoleic acid and lipoxidase. This indicates that beans contain antioxidant.

Table 4 also shows that pure chlorophylls reduce

Table 3. Aerobic (I) and anaerobic (II) degradation of pure chlorophylls *a* and *b* in presence of linoleic acid and a homogenate of fresh beans.

The methods used are those given in Table 2, but either 20 mμ moles of chlorophyll *a* or 13 mμ moles of chlorophyll *b* was used instead of the crude chlorophyll preparation.

3a. Chlorophyll *a*

Period of incubation (min)	Chlorophyll *a* (mμmoles)		Pheophytin *a* (mμmoles)		Total (mμmoles)	
	I	II	I	II	I	II
0	19	20	7	7	26	27
5	14	19	7	8	21	27
10	10	18	6	8	16	26
20	6	19	4	8	10	27
30	4	19	3	7	7	26

3b. Chlorophyll *b*

Period of incubation (min)	Chlorophyll *b* (mμmoles)		Pheophytin *b* (mμmoles)		Total (mμmoles)	
	I	II	I	II	I	II
0	13	12	2	1	15	13
5	10	11	0	2	10	13
10	8	13	0	2	8	15
20	7	13	0	1	7	14
30	4	12	0	2	4	14

Table 4. Inhibition of lipoxidation by crude chlorophyll preparations and pure chlorophylls *a* and *b*.

Soybean lipoxidase (0.1 mg) was used in the standard enzyme assay.

Inhibitor	Fat peroxide formed (mμeq)			
	min incubation			
	5	10	20	30
None	143	273	432	579
30 mμ moles crude chlorophyll	7	4	39	117
28 mμ moles chlorophyll *a*	96	204	302	407
31 mμ moles chlorophyll *b*	110	234	387	502

Table 5. Destruction of chlorophyll during lipoxidation.

Crude chlorophyll (37 mμ moles) was included with 0.1 mg soybean lipoxidase in the standard enzyme assay. Duplicate tubes were used. After incubation fat peroxide was determined in one tube and the other extracted with 3 ml of diethyl ether for chlorophyll determinations.

Period of incubation (min)	Fat peroxide formed (mμeq)	Total concentration chlorophyll and pheophytin (mμmoles)	Conversion chlorophyll to pheophytin (%)
0	4	37	15
5	3	36	66
10	6	38	95
15	18	32	100
20	56	19	100
30	142	12	100

the amount of fat peroxide formed by lipoxidase and linoleic acid alone. This is not due to removal of fat peroxide in reactions with chlorophylls, since 500 mμmoles of fat peroxide (preformed by enzymic peroxidation of linoleic acid) did not react with pure chlorophyll.

The effect of blanching on lipoxidase and catalase in beans. Table 6 shows the effect of blanching on catalase and lipoxidase in beans. Lipoxidase was slightly less heat stable than catalase, and was destroyed in 20 sec.

Chlorophyll changes in other green vegetables. Table 7 shows that during 12 months' storage at −18°C of 5 green vegetables, marked chlorophyll changes occurred only in beans and brussels sprouts. Further, these changes were mainly restricted to conversion of chlorophyll to pheophytin. pH changed little during storage and was not related to chlorophyll changes; for example, little

Table 6. The inactivation of catalase and lipoxidase in beans during blanching.

Whole green beans were blanched in steam at atmospheric pressure for various times and immediately cooled in iced water. Homogenates were prepared for determinations of catalase by the A.O.A.C. (1960) method, and of lipoxidase.

Time of blanch (sec)	Lipoxidase (% of original activity)	Catalase (% of original activity)
5	71	74
10	48	57
15	12	18
20	0	3
25	0	0
30		0

chlorophyll degradation is not related to natural pH, and neither pH nor titratable acidity of beans showed significant trends during 22 months' storage at −18°C. The possibility cannot be ignored that chlorophyll reacts with specific acids in beans, but the present work demonstrates an alternative mechanism. Chlorophylls in crude chloroform extracts of fresh beans were found to become degraded to pheophytins in the presence of linoleic acid and an enzyme capable of promoting fat peroxidation (lipoxidase). The reaction is anaerobic and requires an intermediary present in the crude preparations, since the reaction does not occur with pure chlorophylls.

Table 7. The pH of some green vegetables and chlorophyll degradation during frozen storage.

Whole beans and peas, halved brussel sprouts (¼-inch diameter), quartered green capsicum, and silver beet cut into 1 in. strips were blanched for 2 min in boiling water. Chlorophylls and pH were determined in one sample and a further sample frozen and stored at −18°C for 12 months prior to making these determinations.

Vegetable	pH of a homogenate		Chlorophylls			
			After blanching		After storage	
	After blanching	After storage	Total chlorophyll + pheophytin (μmoles/100 g)	Conversion of chlorophyll to pheophytin (%)	Total chlorophyll + pheophytin (μmoles/100 g)	Conversion of chlorophyll to pheophytin (%)
Beans	5.6	5.7	15.7	8	14.9	68.3
Brussels sprouts	6.2	6.2	9.8	9	8.9	82.9
Green capsicum	5.4	5.5	15.1	0	14.7	4.1
Peas	6.1	6.1	21.3	0	22.3	6.3
Silver beet	6.3	6.3	47.9	2	46.4	4.9

chlorophyll change occurred in capsicum, the most acid vegetable, whereas marked pheophytin formation occurred at the relatively high pH of brussels sprouts.

DISCUSSION

The work confirms the findings of earlier workers that color deterioration is related to the extent of chlorophyll conversion to pheophytin. It also shows that at least two kinds of color degradation occur. The first to develop in storage is associated with conversion of chlorophyll to pheophytin. The second kind, observed after 12 months' storage at −18°C, is associated with progressive destruction of chlorophylls and pheophytins.

The role of acids in determining the high rates of chlorophyll conversion to pheophytin in beans is not clear. Nonspecific acid effects are eliminated since comparison with other green vegetables showed that chloro-

There is some evidence that beans contain an antioxidant, which would account for the twelve months' delay that occurs before the onset of fat peroxidation in stored frozen beans. Crude chloroform extracts of fresh beans incubated aerobically in the presence of linoleic acid and lipoxidase also show a lag phase that is not evident when purified preparations of chlorophylls and pheophytins are used. In all these instances, however, it is significant that simultaneous destruction of chlorophylls and pheophytins coincided with the onset of fat peroxidation, a result in keeping with the observations of Strain (1941).

Since the work demonstrates that both kinds of chlorophyll degradation in stored frozen beans can also occur *in vitro* in

chloroform extracts of fresh beans when these are incubated in the presence of linoleic acid and lipoxidase, it is likely that the degradation reactions are closely linked with those of fat peroxidation. The latter are known to proceed in a series of steps involving initiation, propagation, and termination (Twigg, 1962). Since a free radical is produced during the anaerobic initiation step, it is postulated that this free radical reacts with the as yet unidentified intermediate and causes the degradation of chlorophyll to pheophytin; but the aerobic propagation step, which involves reaction of a free radical with oxygen to produce a peroxy radical, and finally a fat peroxide, is considerably inhibited by an antioxidant present in beans. This antioxidant is evidently slowly exhausted during frozen storage, and, after twelve months, chain propagation commences and both chlorophylls and pheophytins are destroyed, apparently by some aerobic intermediate of the reaction chain.

Lipoxidase was used in this work as a convenient means to peroxidize fats. However, although this enzyme occurs naturally in fresh beans it was inactivated as quickly as catalase by blanching. It is unlikely to persist in commercially frozen bean packs, and indeed no lipoxidase activity was detected in more than thirty samples. However, Twigg (1962) lists at least five other ways in which fat peroxidation may be initiated, and blanching, for example, may account for the fat peroxidation observed after twelve months' storage at −18°C.

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Color Deterioration in Frozen French Beans (Phaseolus vulgaris). 2. The Effect of Blanching

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(Manuscript received December 21, 1963)

SUMMARY

The effect of blanching on the conversion of chlorophylls to pheophytins and on their oxidation during the blanching and subsequent storage of green beans at −10°C was examined. Some conversion of chlorophylls to pheophytins occurred during blanching but there was no oxidation. During storage at −10°C, both conversion and oxidation occurred. Two effects of blanching were distinguished: firstly, as enzymes were inactivated, the rates at which the chlorophylls underwent changes in storage at −10°C diminished; secondly, with blanch periods longer than necessary for inactivation of peroxidase and catalase the rates of chlorophyll change increased progressively during frozen storage. Under the blanching conditions used, a blanching period of 45 sec to 1 min resulted in the most stable product.

INTRODUCTION

Color deterioration in stored frozen beans is closely related to changes in the chlorophylls. Mackinney and Weast (1940) and Dietrich *et al.* (1957, 1959a, 1959b) believed that such changes were restricted to the conversion of chlorophylls to pheophytins, but Walker (1964) has shown that oxidation also occurs in commercial bean packs stored for 12 months at −18°C. He adduced evidence that although conversion and oxidation reactions were both closely linked with fat peroxidation, the enzyme catalyst of fat oxidation, lipoxidase, was not active in blanched beans. Nevertheless, he pointed out that peroxidation of fats may be initiated in several ways, e.g., by blanching. This paper reports investigations on changes occurring in chlorophylls in green beans during blanching and in subsequent frozen storage.

MATERIALS AND METHODS

Preparation of the beans. Beans, var. Hawkesbury Wonder, grown at Tweed Heads, N.S.W., and picked at 9 A.M., arrived in Sydney by air at 5 P.M. During this time the average temperature of the beans was 18°C. The beans were stored overnight at 4°C and prepared for blanching next morning. Crosscut beans were blanched in boiling water for the required period (using a volume of water and a heat input that prevented any cessation of boiling due to the addition of the beans), immediately cooled in water (15°C), and packed into polyethylene bags. The beans were frozen in dry ice and then stored at −10°C until analyzed for chlorophylls. The methods used for the determinations of chlorophylls and pheophytins are described in a previous paper (Walker, 1964).

Determination of "hydroperoxidase" activity. After Theorell (1951), catalase and peroxidase enzymes are referred to collectively as hydroperoxidase. Its activity was determined by the AOAC (1960) catalase method and also by measuring the rate of guaiacol peroxidation as follows: chopped beans were placed to a depth of ½ in. in a 6 × ⅝-in.-diameter test-tube, and covered to a depth of 1 in. with a mixed reagent consisting of equal parts by volume of 1% (w/v) guaiacol in 50% (v/v) ethanol and 5 volume hydrogen peroxide. The tube was shaken frequently and the time taken for the solution to brown was noted. Previous study of the kinetics of the reaction has established that this time is inversely proportional to enzyme activity. Unblanched tissue caused the solution to brown within a few seconds.

RESULTS

Changes in chlorophylls during blanching. Fig. 1 shows that the amount of chlorophyll converted to pheophytin increased approximately linearly with time of blanch up to 3 min, but that with longer blanch times the relative amounts further converted progressively diminished. Analysis for total chlorophyll and pheophytin content showed that the original content of 4.6 μmoles per 100 g beans was unaffected by blanching.

Inactivation of hydroperoxidase during blanching. The results in Table 1 show that both catalase and/or peroxidase were more than two-thirds

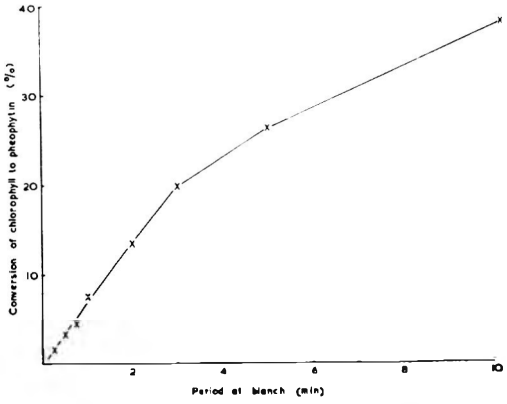

Fig. 1. The conversion of chlorophyll to pheophytin during the blanching of beans.

inactivated during 20-sec blanching, and almost completely inactivated in 30 sec. No enzyme activity was detected in beans blanched for 45 sec or longer.

Changes of chlorophylls at −10°C. Fig. 2 shows the proportion of total chlorophylls that remained in beans subjected to various blanching treatments, as a function of storage time. Loss of chlorophyll after 20 days' frozen storage at −10°C was greatest in unblanched beans, the rate of oxidation being constant over the whole period, as evidenced by the straight-line relation between storage time and the percentage of chlorophyll retained in the beans. No loss occurred over this period in beans blanched for 45 or 60 sec.

Fig. 2 also shows that although blanching times of 20 and 30 sec resulted in lower rates of oxidation and lower total losses of chlorophyll than occurred in unblanched beans over the same period, the rates of oxidation did increase very slightly during storage. The 20-sec treatment resulted in a greater total loss of chlorophyll and a greater

Table 1. The inactivation of catalase and peroxidase during the blanching of beans.

Blanch time (sec)	% of activity of fresh beans	
	Catalase	Peroxidase
0	100	100
20	32	25
30	<1	<1
45	0	0
60	0	0

final oxidation rate than did the 30-sec treatment.

For blanching times greater than 45–60 sec, two distinct features are evident in the curves (Fig. 2). Firstly, there was an initial lag period during which no loss of chlorophyll occurred, this period being longest for the 2-min blanching and becoming progressively less with increased time of blanching, until it was almost absent after 10 min of blanching. Secondly, after the initial lag there was a progressive increase in the rate of oxidative loss with length of storage, this effect being more marked the longer the blanching time. Although even the longest blanching time (10 min) resulted in less loss of chlorophyll after 20 days' storage than occurred in the unblanched beans, the rate of oxidative loss exceeded that of the unblanched beans by about the 14th day. Extrapolation of the curves beyond the period actually studied suggests that by the 23rd day beans that had been blanched for 10 min would have suffered a total loss of chlorophylls even greater than the unblanched beans. A similar trend is apparent for the 5-min treatment curve, but because of the longer initial lag period the cross-over point would have occurred much later.

Fig. 3 shows the influence of blanching time on the apparent conversion of chlorophyll to pheo-

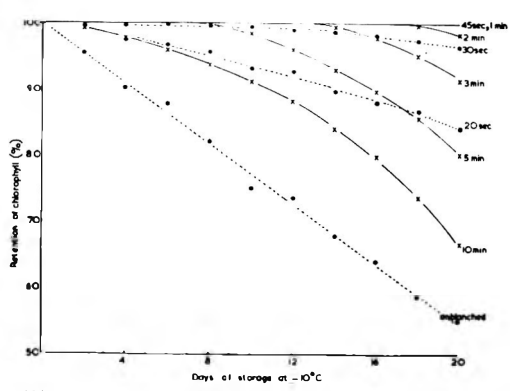

Fig. 2. The oxidation of chlorophylls during storage at −10°C of beans blanched for the periods shown. Broken lines are used for blanch times of 30 sec and less.

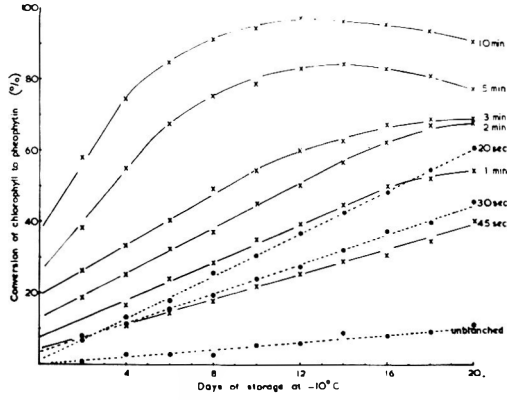

Fig. 3. The apparent conversion of chlorophyll to pheophytin during storage at −10°C of beans blanched for the periods shown. Broken lines are used for blanch times of 30 sec and less.

phytin during storage of the beans at −10°C for 20 days. The initial proportion of chlorophylls converted was fixed by the duration of blanching (see Fig. 1), and the rates at which further conversion appeared to occur during storage, indicated by the slopes in Fig. 3, also varied with blanching time.

In general, the apparent conversion rates were characteristic of the blanching treatments, and each remained constant over the first four or five days. For blanching treatments of 45 sec or less these rates were maintained over the whole storage period. Unblanched beans showed the lowest rate of conversion (0.4% per day), but for treatments of 20, 30, and 45 sec, the highest conversion rate was associated with the shortest blanching time, the rate diminishing with longer blanch time. In consequence, although after 3–4 days the percentage conversion of chlorophyll appeared approximately equal for these treatments, after 20 days the 45-sec blanch had resulted in the least proportion of pheophytin. Beans subjected to a 20-sec blanch showed a higher degree of conversion at the end of the experiment than those blanched for 30 sec.

For blanching periods in excess of 45 sec the conversion rates in the first (linear) phase were greater the longer the period of blanching. These rates gradually diminished on further storage, the decrease occurring sooner and more rapidly as the time of blanching was extended. Beans blanched for 5 and 10 min passed through a stage at about 15 and 13 days, respectively, when the apparent rate of conversion was zero. After this, the relative amount of chlorophyll actually increased and the conversion rate appeared to be negative. Reversion of pheophytin to chlorophyll being unlikely, it is clear that at this stage the rate of loss of pheophytins through oxidation exceeded that of the chlorophylls.

DISCUSSION

The present work confirms the results of Mackinney and Weast (1940) and Dietrich *et al.* (1957, 1959a, 1959b) that chlorophylls are converted to pheophytins when beans are blanched. These changes continue during frozen storage, when oxidation of chlorophylls also occurs. Walker (1964), after showing that both phenomena are closely linked with fat peroxidation, postulated that chlorophylls are converted to pheophytins during the anaerobic initiation stage of fat peroxidation, and that when the reaction chain propagates, chlorophylls are oxidized.

Fat peroxidation may be initiated enzymically or by heat, and, in accord with the above hypothesis, the observed effects of blanching on chlorophyll changes may be considered to be the result of two mechanisms. In the first stage, represented by unblanched beans and beans blanched for 20 and 30 sec in boiling water, enzymes were either not inactivated or only partially inactivated, and changes in chlorophylls were diminished in proportion to the degree of inactivation. In the second stage, other reaction systems were initiated by heating; this is seen most clearly in beans blanched for 45 sec or longer, since the results are not obscured by enzyme effects.

Where heat initiation was involved, the oxidation of chlorophylls during storage at −10°C increased with the period of blanch (Fig. 2). However, the initial lag period before oxidation commenced has to be explained. A similar lag observed before chlorophylls were oxidized in commercial bean packs stored at −18°C was attributed to the presence of antioxidants (Walker, 1964); these only affected aerobic reactions, and did not prevent the anaerobic conversion of chlorophylls to pheophytins. In the present work chlorophyll conversion to pheophytin during the oxidation lag phase was linear with respect to time of storage, and the rate increased with the period of blanch. If pheophytin formation was related to fat oxidation, an acceleration in the rate of these reactions would result in more rapid utilization of antioxidants and explain the shortening of the lag phase before oxidation commenced. Once oxidation of the chlorophylls starts, however, the rate of conversion to pheophytin apparently declines. This could occur only through preferential oxidation of pheophytin since there was no synthesis of chlorophyll.

In the phase of "enzyme inactivation" chlorophyll oxidation commenced immediately on storage and the oxidation rates were reduced as enzymes were inactivated. In contrast to oxidations, however, the conversion of chlorophyll to pheophytin was least in unblanched beans. This would be explained if pheophytins were oxidized preferentially, as occurred during heat-initiated oxidations. However, pheophytins

accumulated even when chlorophyll oxidation did occur, as in beans blanched for either 20 or 30 sec. The reasons are obscure, but such a result might occur as an effect of residual enzyme- and heat-initiated processes, or by uncoupling of chlorophyll conversion and oxidation reactions.

Thus this work provides clear evidence that changes in chlorophylls in beans were initiated either enzymically or by heat. When it is necessary to minimize color changes during frozen storage a blanch time should be selected such that enzymes are inactivated without undue heat initiation of other systems. If such blanch time is used, color deterioration in storage can be measured as chlorophyll conversion to pheophytin. With shorter or longer blanch times, however, chlorophyll oxidation will become a significant factor in the overall assessment of color.

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Origin of Hydrogen Sulfide in Heated Chicken Muscle

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(Manuscript received October 24, 1963)

SUMMARY

About 90% of the hydrogen sulfide (H_2S) produced by heated chicken muscle comes from muscle protein. To identify specific H_2S precursors, the identity, quantity, and H_2S-producing capability of sulfur compounds in chicken muscle were investigated. The only sulfur compounds found in muscle nonprotein were methionine, taurine, and glutathione. Of these, only glutathione produced H_2S. Therefore the principal H_2S precursor in chicken muscle nonprotein is sulfur, occurring as cystine and/or cysteine in the tripeptide glutathione. Since the only sulfur compounds reported in chicken muscle protein are methionine, cystine, and/or cysteine, and since methionine does not produce H_2S, the sulfur in the H_2S produced by muscle protein must also come from cystine and/or cysteine. Glutathione gives off H_2S about 180 times as fast as does chicken muscle protein, but, because there is approximately 1 to 2 thousand times as much protein as glutathione in muscle, protein is the principal H_2S precursor. Since cystine plus cysteine sulfur in protein and in glutathione is the only H_2S precursor, the rate of H_2S evolution from heated chicken muscle can be approximately predicted from its cystine content.

INTRODUCTION

The small amount of hydrogen sulfide (H_2S) formed and volatilized when chicken is heated may be sufficient to influence aroma (Bouthilet, 1951; Pippen and Eyring, 1957; Lineweaver and Pippen, 1961). For evaluating the importance of H_2S to the aroma and flavor of cooked chicken, it is desirable to know its origin and rate of formation. Sadikov *et al.* (1934) reported that muscle protein is the precursor of H_2S, and Bouthilet (1951) suggested that the precursor is glutathione. Kazeniac (1961) showed that both skin and meat yield H_2S on heating. However, none of these reports gives the relative amounts of H_2S produced by the protein and nonprotein fraction of muscle or by specific substances in muscle such as glutathione.

The present study was made to determine the source(s) of H_2S in chicken muscle. Observations were made on whole muscle, muscle protein, muscle nonprotein, taurine, methionine, cystine, cysteine, and glutathione boiled in pH 6.5 buffer. Comparisons were also made of the quantities of H_2S boiled out of: chicken leg muscle, its water-insoluble and water-soluble fractions, and the heat-coagulable and non-heat-coagulable parts of the water-soluble fraction.

MATERIALS

Reagents. The reagents used are adequately described in the literature cited, except the following:

a) *Distilled water.* Prince (1955) stressed the importance of using highly purified water in determining trace amounts of H_2S. Therefore water was triple distilled from glass (second distillation from $KMnO_4$) and finally boiled at least 15 min to expel oxygen. During the final boiling and cooling period, and in subsequent use, nitrogen was used to expel oxygen and prevent its re-entry. This specially prepared water, hereinafter referred to simply as distilled water, was used for all water requirements including reagent preparation.

b) *Glutathione.* Reduced glutathione (GSH) and oxidized glutathione (GSSG) from commercial sources were used. Assays for sulfhydryl by the method of Sokol *et al.* (1959) showed that the GSH sample contained 1.04 moles of sulfhydryl/mole, and that the GSSG sample contained less than 0.001% sulfhydryl. In addition, it was deter-

mined that the GSH and GSSG samples were chromatographically pure.

c) *Amino acids.* Taurine, cysteine (free base), cystine, and methionine were used as received from commercial sources.

d) *pH 6.5 buffer.* Buffer was prepared just before use by mixing 160.0 ml of $M/15$ Na_2HPO_4 and 340.0 ml of $M/15$ KH_2PO_4. The pH was determined and, if necessary, adjusted to precisely 6.50 by addition of $1N$ NaOH.

METHODS

Chicken muscle samples. Chilled, ready-to-cook fowl carcasses were bagged in polyethylene on the day of processing and held at −10°F. Leg and breast muscles were cut from four partially thawed, skinned carcasses. Readily separable fat was trimmed away and discarded. Leg muscles from the four carcasses were combined, minced in a Hobart food chopper in a room at 34°F, and thoroughly mixed. Breast muscles were similarly treated. The meat samples (divided into 25- and 50-g portions) were held in polyethylene bags at −30°F until used.

Water-soluble and water-insoluble fractions. Ground leg muscle (62.5 g) was mixed with 130 ml of chilled (34–40°F) distilled water and allowed to stand 20 min in a room at 36°F. The mixture was then spun 20 min at 2500 rpm in a refrigerated (40°F) centrifuge, and the supernatant was decanted. This extraction procedure was repeated 6 times. H_2S was determined on the insoluble residue and on the combined water extracts by the distillation-trap method described below (see the section on hydrogen sulfide determinations).

Protein and nonprotein fractions. The procedure of Tallan *et al.* (1954) was adapted to this study. Muscle samples (20.0 g) and 200 ml of 1% picric acid were mixed in an Omnimixer for 3 min at full speed, with the mixing cup immersed in ice water. After standing 10–15 min, the sample was centrifuged 15 min in a refrigerated (40°F) centrifuge at 2500 rpm and the picric acid extract was then decanted.

After being washed 4–5 times with 100-ml portions of acetone, the protein was spread in a thin layer in a large crystallizing dish and dried overnight at room temperature in a stream of nitrogen. It was held at −30°F until used for H_2S determination.

Picric acid was removed from the extracts by the detailed methods of Stein and Moore (1954) and Tallan *et al.* (1954). The picrate-free extracts were stored at −30°F until the quantity of H_2S produced on heating them could be determined and the sulfur compounds in them could be estimated chromatographically.

Chromatography. To determine the identity and quantity of sulfur compounds in muscle nonprotein, the concentrated picrate-free muscle extracts were chromatographed on a Beckman/Spinco Model 120 amino acid analyzer under conditions recommended in the manufacturer's instruction manual.

Determination of glutathione. Glutathione was determined in the concentrated picrate-free muscle extracts (nonprotein fraction). Only about 50% of the glutathione could be converted to glutathione-*S*-sulfonate ($GSSO_3H$) by following the procedure recommended in the amino acid analyzer manual; therefore, the following modified procedure was developed:

The concentrated extract was transferred to a 100-ml beaker with 30 ml of distilled water containing 10 ppm copper as cupric nitrate. After adjusting the pH to 7.5 with $1N$ NaOH, the solution, with oxygen bubbling through it, was stirred 4 hr at room temperature. Then 2.6 ml of freshly prepared $0.5M$ Na_2SO_3 was added, the pH was readjusted to 7.5 with $2N$ HCl, and the solution was again stirred 4 hr at room temperature with oxygen bubbling through it. Then the pH was adjusted to 2.2 with $2N$ HCl, and the solution was diluted with water to 50.0 ml. One volume of this was diluted with an equal volume of the pH 2.2 citric acid buffer used for application of samples to the amino acid analyzer. Two to 6 ml of the resulting solution was applied to the column for chromatography.

Peaks attributable to glutathione in untreated (unoxidized) muscle extracts did not appear on chromatograms of extracts that had been oxidized by this procedure; instead there was a corresponding appearance of the $GSSO_3H$ peak. Furthermore, the modified procedure, when applied to GSH and GSSG, gave chromatograms showing a single peak corresponding to $GSSO_3H$.

To provide a standard, 16.0 mg of GSH dissolved in 30 ml distilled water containing 10 ppm copper was treated by the modified procedure and chromatographed. Peaks corresponding to $GSSO_3H$ in muscle extracts were compared to the standard $GSSO_3H$ peak to determine the quantity of glutathione in the muscle extracts. Standards and muscle samples were run in duplicate.

Hydrogen sulfide determinations. The methylene blue method of Sands *et al.* (1949) as modified by Prince (1955) was adapted to this study. Careful attention was given to the latter's instructions concerning purity of water, cleansing of glassware, and dissolution of any zinc sulfide adhering to glass surfaces of the trap. Optical density of the methylene blue color was determined on a Beckman Model DU spectrophotometer at 745 mμ with the red-sensitive phototube and a slit width of 0.045–0.05 mm.

H_2S was recovered for determination by two methods: the reflux-trap method, and the distillation-trap method.

Used in the reflux-trap method was the apparatus shown in Fig. 1. The sample being analyzed,

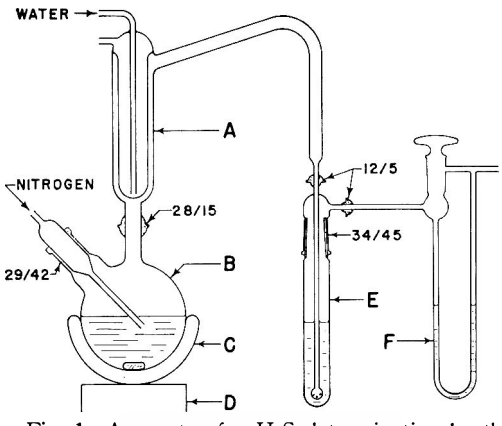

Fig. 1. Apparatus for H_2S determination by the reflux-trap method. A) Modified Friedrichs condenser. B) Modified 1-L flask. C) Heating mantle. D) Motor for magnetic stirrer. E) Trap, 32 mm OD × 23 cm long (bottom of trap to top of inner joint), containing 5 ml of 20% zinc acetate, 45 ml distilled water, and 1 small drop of acetic acid. F) Flowmeter operated at a pressure differential of 70–72 mm of water. (Fractions designate the sizes of ground-glass joints.)

500 ml of pH 6.5 phosphate buffer, a squirt of Dow-Corning Antifoam A spray, and a glass- or plastic-covered magnetic stirring bar were placed in boiling flask B. Then, with the stirrer operating and all components in place, high-purity nitrogen was passed through the system at a rate causing a pressure difference of 70–72 mm of water on the flowmeter (Pyrex brand, using the ¼-mm orifice). After 30 min at room temperature, the zinc acetate trap was replaced and the heating mantle turned on. After the 11 min required to reach the boiling point, the system was kept boiling under gentle reflux throughout the run. The second zinc acetate trap was kept in place during the warm-up period plus 30 min boiling time. Thereafter, traps were normally replaced at 30-min intervals. Reagents required to develop the methylene blue color were added directly to the traps.

In the distillation-trap method, the sample, in 500–650 ml distilled water, was boiled while stirred with a magnetic stirrer in the 1-L flask of Fig. 1. The condenser, shown in Fig. 1, was arranged for distillation. The distillate, approximately 300 ml, was collected in a receiver having an inlet tube extending nearly to the bottom. A zinc acetate trap (E, Fig. 1) was attached to the receiving flask to collect any H_2S not retained in the distillate. The system was kept under slight pressure with nitrogen, to prevent the distillate from sucking back. The total H_2S produced during distillation was determined from the H_2S in the trap and in an aliquot of the distillate.

RESULTS

H_2S produced by chicken muscle. H_2S was not detected during the 30-min period preceding heating when the raw muscle, in water or buffer, was being analyzed at room temperature (zero boiling time, Fig. 2). However, muscle boiled for 2–2½ hr produced H_2S at rates essentially constant for each condition (Fig. 2). Even after boiling 25 hr at pH 6.5, H_2S continued to evolve at about half the rates shown in Fig. 2.

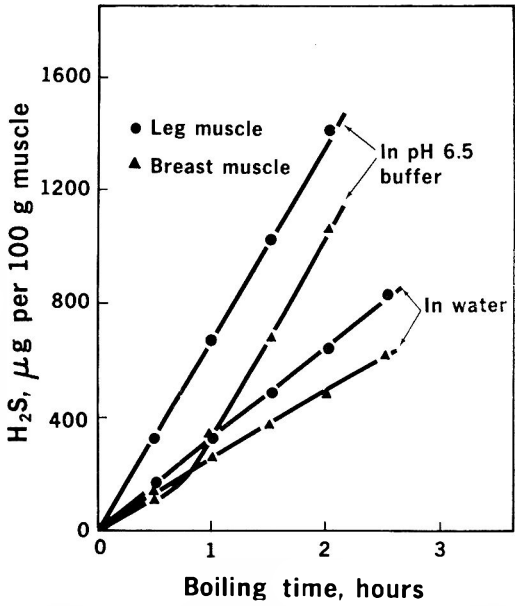

Fig. 2. H_2S produced by chicken muscle boiled in distilled water and in pH 6.5 buffer as determined by the reflux-trap method. Values are averages of duplicates except for single determinations at 2½ hr. The pH of muscle and water mixtures: before heating, 6.07 for leg and 5.77 for breast; after heating, 6.50 for leg and 6.16 for breast.

Initial and final pH values are given in the legend of Fig. 2 for muscle boiled in water, since pH increased under this condition. Leg muscle in water had higher comparable pH values than breast muscle, and evolved H_2S faster. Also, leg and breast muscles evolved H_2S at greater rates when buffered at pH 6.5 than they did in water, where their average pH values were 0.2–0.5 less than 6.5. The faster rate of H_2S evolution at higher pH values is consistent with the results of Kazeniac (1961).

The slower H_2S production from breast muscle than from leg muscle, during the first 0.5–1 hr in buffer at pH 6.5, was observed in each of 4 separate determinations. This, too, may be a pH effect, reflecting the possibility that more time was required for the buffer to become effective in breast muscle than in leg muscle because the buffer had to raise breast muscle 0.73 pH unit and leg muscle only 0.34 pH unit.

To reduce pH variation, H_2S determinations, unless stated otherwise, were carried out in buffer at pH 6.5.

H_2S produced by water-soluble and water-insoluble fractions. To first establish whether protein was the main source of H_2S in an unbuffered system, we compared the water-insoluble and water-soluble fractions of chicken muscle and the heat-coagulable and non-heat-coagulable portions of the water-soluble fraction. The quantity of H_2S produced by the water-insoluble fraction of leg muscle was twice that from the water-soluble fraction (Table 1). The coagulum that appeared when the water-soluble fraction was boiled, was filtered off following H_2S determination. Hydrogen sulfide produced by reboiling the coagulum was substantially greater than H_2S produced by reboiling the filtrate (Table 1). The water-insoluble fraction and coagulum account for 401 μg, or 87% of the total amount of H_2S produced (461 μg). Since the water-insoluble and the heat-coagulable fractions are essentially protein, protein must be the main source of H_2S.

Table 1. H_2S produced by boiling the water-insoluble and water-soluble fractions of chicken leg muscle and by reboiling the heat-coagulable and non-heat-coagulable portions of the water-soluble fraction.

Fraction	H_2S [a]
	μg
Water-insoluble (from 100 g muscle)	311
Water-soluble (from 100 g muscle)	150
Coagulum from water-soluble fraction	90
Filtrate from coagulum	36

[a] Determined by the distillation-trap method. Values are averages of two to three determinations.

H_2S produced by muscle protein and muscle nonprotein fractions. Fig. 3 shows the amount of H_2S produced by protein and nonprotein chicken muscle fractions obtained by treating the muscle with 1% picric acid. Muscle protein, like muscle, did not produce H_2S until heated, and then leg and breast muscle proteins produced it at about equal, uniform rates (Fig. 3). During 2 hr of boiling, leg and breast muscle proteins respectively produced 75% and 84% as much H_2S as did leg and breast muscles (*cf.* Fig. 2). In contrast, muscle nonprotein fractions did not produce detectable H_2S during the first 2 hr of boiling (Fig. 3). Thereafter they yielded H_2S, but at a much lower rate than protein.

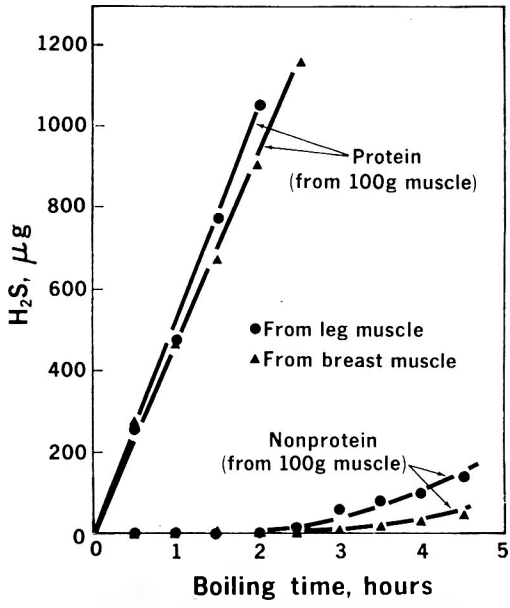

Fig. 3. H_2S produced by protein (insoluble in 1% picric acid) and nonprotein (soluble in 1% picric acid) fractions of chicken muscle boiled in pH 6.5 buffer, as determined by the reflux-trap method. Results are based on the analysis of 20-g muscle samples. Values are averages of 2 or 3 determinations except the last 3 values for leg muscle nonprotein, which are single determinations.

H_2S produced by glutathione and sulfur amino acids. Chromatography of the nonprotein fraction of leg and breast muscle revealed the sulfur compounds, taurine, methionine, and glutathione, but no cystine or cysteine. Taurine boiled in pH 6.5 buffer for 2 hr and methionine for 3 hr did not produce detectable amounts of H_2S. Therefore, a study was made of H_2S produced by glutathione and of the quantity of glutathione in chicken muscle.

Chromatograms of muscle extracts that had not been treated to convert glutathione to the sulfonate showed a peak eluting at the same volume as $GSSO_3H$ and equivalent to as much as 3 mg of GSH/100 g muscle. Therefore the quantity of GSH indicated by the $GSSO_3H$ peak was reduced by 3 mg to give the values shown in Table 2. The method seemed reliable, since the addition of a known quantity of GSH to leg muscle was recovered quantitatively (Table 2).

During 2 hr boiling, 20 mg GSH (approximately the maximum in 100 g muscle) produced 130 μg H_2S, 100 g leg muscle produced 1420 μg, and 100 g breast muscle produced 1070 μg

Table 2. Glutathione content of chicken muscle.

Determination no.	Glutathione (as GSH) (mg/100 g muscle)	
	Breast muscle	Leg muscle
1	8.6	17.2
2	9.3	18.1
3	.....	39.0[a]

[a] A single determination in which GSH was added to muscle at a level of 20 mg/100 g.

(Fig. 4). Hence, the 18 mg glutathione in 100 g leg muscle (Table 2), if present as GSH, would produce only 8.5% of the total amount of H_2S produced by leg muscle. Similarly, the glutathione present in breast muscle as GSH would produce only 5.6% of the total H_2S. If a substantial portion of glutathione in muscle is GSSG, the fraction of the total H_2S originating from glutathione would be even less. Chromatographic analysis showed that glutathione existed as GSSG in the muscle extracts as prepared for H_2S analysis. Hence the curves for H_2S produced by muscle nonprotein fractions lie closer to the GSSG than the GSH curve (Fig. 4).

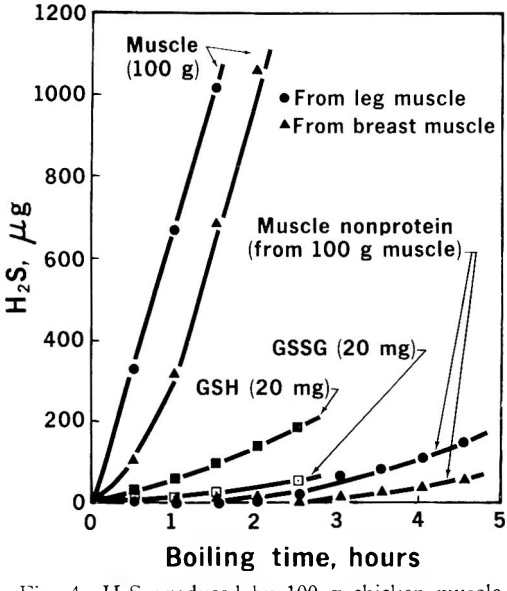

Fig. 4. H_2S produced by 100 g chicken muscle, muscle nonprotein equivalent to 100 g muscle, and glutathione approximately equivalent to 100 g muscle. Samples were boiled in pH 6.5 buffer, and H_2S was determined by the reflux-trap method. Values for glutathione are single determinations. Muscle and muscle nonprotein data are from Figs. 2 and 3.

Since 20-g samples of muscle were analyzed, only about 1.8 to 3.5 mg GSSG was present in the nonprotein fractions. This amount of GSSG would produce only about 1 μg or less of H_2S per 30 min during the first 1½–2 hr. The method was not sensitive to less than 1 μg H_2S, hence none could be detected during the first 1½–2 hr from the nonprotein fractions (Fig. 4). The rates at which the nonprotein fractions produced H_2S increased with boiling time, hence readily measurable amounts of H_2S were eventually produced by the nonprotein fractions.

DISCUSSION AND CONCLUSIONS

The results show conclusively that protein is the principal source of H_2S in heated chicken muscle.

The relative efficiency with which muscle and some of its components produced H_2S was calculated (Table 3). On a dry-weight basis (Table 3, column I) glutathione, as GSH, produced H_2S about 160 times as fast as did breast muscle protein. However, the protein/glutathione dry-weight ratios were about 1250 for leg muscle and about 2500 for breast muscle. Hence the 1.25-to-2.5-thousandfold greater weight of protein than glutathione more than nullifies the 160-to-180-fold greater rate of H_2S production from glutathione, and makes protein the principal H_2S precursor. It seems unlikely that glutathione would occur at a level high enough—it would have to be about 150 mg/100 g muscle, compared to the observed level of about 18 mg/100 g—to rival muscle protein as the major H_2S precursor.

Since there are several types of sulfur linkages in the various muscle fractions, it is not surprising that the rate of H_2S production is not closely related to the total sulfur in the fractions (Table 3, column II).

Beach *et al.* (1943) found that methionine and cystine sulfur account for essentially all sulfur in chicken muscle protein. Methionine did not produce H_2S; hence cystine and cysteine, in muscle protein and in the tripeptide glutathione, must be the H_2S precursors in chicken muscle. Muscle protein produces more H_2S than muscle nonprotein does because there is 150–300 times as much cystine and cysteine in muscle protein as there is in muscle nonprotein. Only about 1% of cystine plus cysteine sulfur is transformed per hr to H_2S; thus it is understandable that production of H_2S continues for many hours.

It is evident from the results for glutathione, cystine, and cysteine (Table 3) that the

Table 3. Rate of H_2S production from chicken muscle and other parent components boiled in buffer (pH 6.5). Rates calculated to the bases: parent component dry weight, total sulfur in parent component and cystine plus cysteine sulfur in parent component.

Parent component	H_2S, μg (av hourly quantity produced), per mg of: I Parent component (dry weight)[a]	II Total sulfur in parent component[b]	III Cystine plus cysteine sulfur in parent component[c]
Reduced glutathione (GSH)	3.65	35.03	35.03
Oxidized glutathione (GSSG)	1.01	9.66	9.66
Cysteine	4.96	24.39	24.39
Cystine	3.10	11.63	11.63
Muscle, leg	0.037	3.53	11.44
Protein, leg muscle	0.023	2.92	6.74
Nonprotein, leg muscle	0.0085	0.20	11.04
Muscle, breast	0.027	2.63	8.68
Protein, breast muscle	0.021	2.52	5.96
Nonprotein, breast muscle	0.0041	0.13	11.05

[a] The following dry-weight values for leg and breast components, respectively, were used: whole muscle, 25% and 25%; protein, 22.6% and 22.5%; nonprotein, 2.4% and 2.5%.

[b] The rates were calculated using the following total sulfur values observed for (wet) leg and breast muscles respectively: whole muscle, 0.26% and 0.26%; protein, 0.18% and 0.18%; nonprotein, 0.10% and 0.08%.

[c] Muscle protein and glutathione were assumed to be the only significant sources of cystine and cysteine sulfur. The following values for leg and breast muscle components, respectively, were used: dry muscle protein, 1.3% and 1.3% cystine (as reported by Beach *et al.*, 1943); glutathione, 9.0 and 17.7 mg/100 g wet muscle (average of values reported in Table 2).

rates at which cystine and cysteine produce H_2S will be influenced by the type of peptide linkage and by the sulfhydryl/disulfide ratio. Consequently, the rate at which muscle, or any of its fractions, produces H_2S probably cannot be accurately predicted solely from cystine plus cysteine sulfur content. Nevertheless, the disulfide-sulfhydryl structure is obviously important to H_2S production since the rate of H_2S production per mg cystine plus cysteine sulfur is fairly uniform (Table 3, column III). The values show that muscle and its fractions produce H_2S at 51–98% of the rate found for cystine. Hence, cystine content provides a reasonably good estimate of the H_2S-producing capability of muscle and muscle fractions.

H_2S odor can be detected when present at a level as low as 1.1 μg per liter of air (Fieldner *et al.* 1931). Therefore, it is probable that the concentration of H_2S evolved into air, in the immediate vicinity of boiling chicken meat, is great enough to contribute to aroma because it takes only 100 g of chicken muscle to evolve as much as 9–12 μg of H_2S per minute. However, before we can conclude that H_2S contributes directly to flavor, we must establish that significant quantities of H_2S remain in chicken, or continue to evolve from it, when it is eaten.

H_2S could also contribute to flavor by forming other compounds. For example, we can predict from Barch's (1952) results that reaction between H_2S and some of the carbonyls found in chicken (Pippen *et al.*, 1958; Pippen and Nonaka, 1960) could form flavoring material. The various ways in which H_2S can contribute directly and indirectly to flavor are being investigated.

ACKNOWLEDGMENT

The authors thank Cheryl J. Knapp for assaying authentic glutathione samples, and Helen M. Kivela for carrying out the amino acid analyses.

Reference to a company or product name does not imply approval or recommendation.

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Determining the Activity of a Glucose Oxidase and Catalase Mixed Enzyme

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(Manuscript received August 27, 1963)

SUMMARY

A polarographic method for determining the mixed enzyme glucose oxidase and catalase is presented. By appropriate adjustment of the parameters of the reaction the activity of the catalase can be determined under simultaneous elimination of the glucose oxidase, or simultaneous determination of the total activity of the mixed enzyme and the catalase can be carried out. This makes possible a quick orientation about the contents of hydrogen peroxide and oxygen at arbitrarily chosen time intervals. Furthermore, the method enables one to determine the change of activity when a long-term effect of a sufficient amount of oxygen takes place.

The determination of enzyme activity is a problem frequently met in practice. On the one hand, it may relate simply to an analytical process directed to determining proper quantitative values in preparing an enzyme during fermentation, and in control between the individual steps of the isolation process; or, on the other hand, the question may be of determining the catalytic effect that a certain enzyme under specific conditions exerts upon a given reaction, whereby it cannot only influence the reaction course, but also give a picture of the qualitative and quantitative conditions in the whole system. The latter problem arises in connection with the dose of an enzyme added to certain foods, in which case it is necessary to know in advance the activity of the enzyme in the particular (mostly more complex) biological material.

As is usual in most analyses of natural substances, the methods of determining the enzyme activity are a combination of chemical, biological, and biochemical methods. Our task was to determine the activity of commercial preparations of beta-D-glucopyranosoacerohydrogenase, briefly designated as "glucose oxidase." Said preparations contain, besides glucose oxidase, still another enzyme, in variable quantity, namely the catalase. The method most often employed for determining the activity of glucose oxidase is Warburg's method, currently used in biochemistry for determining the interchange of gases in tissues, cells, or pure solutions of enzymes and substrates. Another method sometimes still used is the acidimetric determination of the increasing content of gluconic acid, which is the final product of glucose oxidation under catalytic action of glucose oxidase.

With the Warburg method, working at constant volume and temperature and measuring alteration of pressure of the gas consumed in the biochemical reaction, one operates under a permanent excess of oxygen. The second method mentioned, based on acidimetric determination of gluconic acid, likewise requires the presence of excess oxygen, since for producing a sufficient and acidimetrically appreciable amount of gluconic acid, glucose oxidase must be allowed to act for a longer time and under an intensive access of oxygen. However, under operational conditions, glucose oxidase is mostly used for removal of oxygen from a confined space, and therefore a medium with limited oxygen content must be considered. This is the fact about the Warburg method that Underkofler (1957) pointed to, suggesting that the determination be carried out in the closed pipette of an Orsath apparatus. However, this modification would itself bring about other disadvantages. The same drawbacks as in the Warburg method also interfere in the acidimetric method.

Besides the aforementioned circumstance of operating at oxygen excess, all the cited methods are encumbered with a further drawback in that none of them affords any picture of the action of catalase upon the reaction medium, thus making it impossible to determine the concentration of peroxide that influences both the activity of the two enzymes and the course of other biochemical reactions in the biological materials. These methods would make sense only when one deals with a pure enzyme preparation. But when a mixed enzyme is present, their use is not justifiable, since they do not determine the activity of the two individual enzymes, the action of which takes its course according to the following summary reaction schemes:

$$\text{I. Glucose} + H_2O + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

$$\text{II. } H_2O_2 \xrightarrow{\text{catalase}} H_2O + \tfrac{1}{2} O_2$$

In the kinetic respect the enzymatic reactions can take their course according to zero or first-order equations. In the first case the reaction runs according to the relation $x = k \cdot t$, from which it follows that the reaction rate remains unchanged. This is true when the substrate is in steady excess. The constant rate is dependent not on the total concentration of the substrate but on the complex of enzyme and substrate; the quantity of this complex remains constant during the whole reaction. A relation has been derived to express numerically the formation of the complex. It is the equilibrium constant of the reaction: enzyme + substrate $\rightleftarrows$ complex (Michaelis and Menten, 1913). This constant is essentially invariable for the given system of enzyme and substrate.

At other conditions, namely with restricted access to substrate, the reaction assumes a quite different course. The dependence of the substrate concentration on the time has a parabolic course, the rate constant being in this case $k = (1/t) ln (a/a - x)$, in which a is initial substrate concentration and x is the decrease of concentration. The dependence of $ln (a - x)$ on the time is linear, and from its graphical representation k can be determined from the slope. All our cases conformed to the latter equation. The content of the substrate, which is glucose, was always in excess, no doubt, but besides it an important limiting factor was oxygen as electron acceptor, the content of which was not constant during the reaction, as we operated at limited air access. From the course of the decrease thereof the rate constants were calculated.

Among the reactions catalyzed by enzymes, no reactions of the second order were observed (Anderson *et al.*, 1946).

Our task was to develop a method that would eliminate the drawbacks of the methods mentioned. For that purpose we applied the polarographic method, permitting a rapid orientation about all the aforesaid factors influencing the given biochemical reactions. For a similar purpose, namely for determining the activity of ascorbate oxidase, the polarographic method was used by Sack *et al.* (1961). For our purpose the following measurements were carried out:

1) Determination of the total activity of the mixed enzyme under limited oxygen access;
2) Determination of the catalase activity separately;
3) Simultaneous determination of both the total activity of the mixed enzyme and catalase;
4) Determination of alteration in activity of the mixed enzyme during a long term with sufficient oxygen.

The single determinations were carried out directly in Kalousek's polarographic vessel (with a water mantle maintained within ± 0.1°C, with separate saturated calomel electrode connected by an agar bridge), as follows:

To a 0.03*M* solution of glucose in 0.1*M* acetate buffer were added a few drops of dextran solution, and the mixture obtained was saturated with air for 15 min. As previously established, this time was sufficient for complete saturation of the solution with atmospheric oxygen. The oxygen content corresponding to the temperature was calculated according to tables for aqueous solutions of gases, and at 23°C was considered equal to 0.85 mg O_2/100 ml. The

concentration of the dissolved substances was too small to influence air solubility significantly. To avoid any misrepresentation of the reaction course due to absorption of atmospheric oxygen during the reaction, the reaction solution was covered with a layer of paraffin oil. After the bubbling-through was ended, the height of the first reduction wave of oxygen, corresponding to the above-quoted content of O_2, was read. In the medium examined, i.e. at pH 3.6–5.6, said reduction wave proceeds within the range of +0.2 to −0.6 volts per standard calomel electrode (SCE). Before adding glucose oxidase the position of the galvanometer at a voltage −0.45 V/SCE was recorded, and all further measurements were performed at this constant voltage.

Under bubbling of air, continued to secure perfect and rapid mixing, the solution of glucose oxidase was introduced in such a manner as to obtain the concentration desired. After a few seconds, bubbling was interrupted, and alteration of the oxygen wave height at said voltage was observed continuously. In this way the course of the biochemical reaction was followed.

The enzyme activity is usually quoted in so-called Sarette units, stating the oxygen consumption under optimum conditions for glucose oxidase action. This mode of evaluation is applicable when the determination is performed under constant excess of oxygen, and therefore the reaction proceeds at constant rate. In our case, where the operation was performed with restricted access of air, this was not the case; the rate of oxygen consumption at any given time was proportional to its concentration. The curve illustrating the biochemical reaction had a logarithmic character conforming to a kinetic equation of the first order. After removal of a major part of oxygen, when the wave height attains already low values, the measurements are encumbered by errors. Nevertheless, the rate constant can be calculated from the reproducible part of the curve. As another criterion of enzyme activity the so-called half-life period can be employed, i.e. the time interval during which the oxygen content falls to half. In applying the enzyme to biological material, where a complete removal of oxygen from the medium is required, the polarographic curve must be followed to zero values of oxygen, from which is determined the time necessary to its complete removal.

The reaction course at 23°C and pH 5.6 is represented in Fig. 1. The commercial

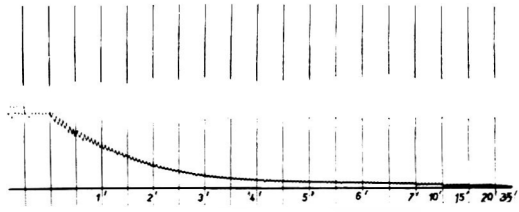

Fig. 1. Limit current height of the first oxygen reduction wave in given time intervals, in the presence of glucose oxidase. Preparation DeeO, 1:5000; pH 5.6; T = 23°C.

preparation DeeO, with reported activity of 1500 S.u., was used for the determinations. All other cases, illustrated in additional graphs, were carried out under the same conditions.

From the polarogram reproduced in Fig. 1, illustrating the drop of limit current of the first reduction wave of oxygen, the content of oxygen in a given time interval can be calculated on the basis of the relation

$$O_t = O_o \frac{A_t}{A_o}$$

O_t = content of O_2 in the time t

O_o = content of O_2 in the air-saturated solution before introduction of the enzyme, i.e. 0.85 mg/100 ml

A_o = limit current height of the first reduction wave before introducing the enzyme (from the galvanometer zero position) at −0.45 V/SCE

A_t = limit current height of the first reduction wave in the time t at −0.45 V/SCE

Fig. 2 shows the course of oxygen consumption and of the logarithm of its concentration in given time intervals. From the data established, the rate constant has been calculated, having the value of $4.32 \cdot 10^{-3}$ sec^{-1} at the given conditions. The constant thus established was not the true rate constant, however, being dependent on the enzyme concentration and pH at which the reaction was performed, but it can be considered as

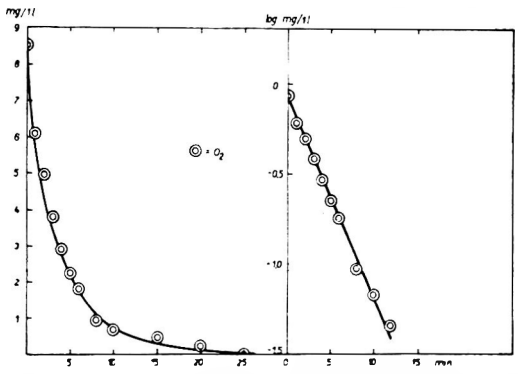

Fig. 2. Plot of oxygen consumption and of the logarithm of its concentration vs. time. Preparation DeeO, 1:10000; pH 5.6; T = 23°C.

reproducible and comparable under the given conditions and for the given system.

The second determination, specifically of the action of catalase, was carried out in a similar way, with the difference that glucose was not added to the reaction medium, and that the solution was bubbled through with nitrogen sufficiently to remove oxygen. A known amount of H_2O_2 was added, and the height increase of the limit current of the first oxygen wave was followed at a constant voltage of −0.45 V/SCE. From the oxygen increase, the decrease of the peroxide was determined by stoichiometric conversion. The values thus obtained indicate that the reaction proceeds according to a kinetic equation of the first order also in this case, and that the rate constant thereof can be calculated correspondingly. The course is shown in Fig. 3. As shown in Fig. 4, the dependence of the logarithm of the H_2O_2 concentration on the time has a linear course. Hence, the reaction proceeds according to an equation of the first order.

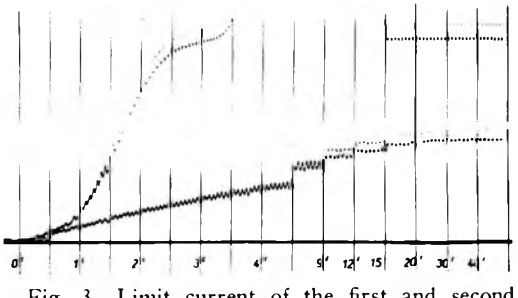

Fig. 3. Limit current of the first and second oxygen reduction waves under action of glucose oxidase on hydrogen peroxide. Preparation DeeO, 1:3000; pH 5.6; T = 23°C.

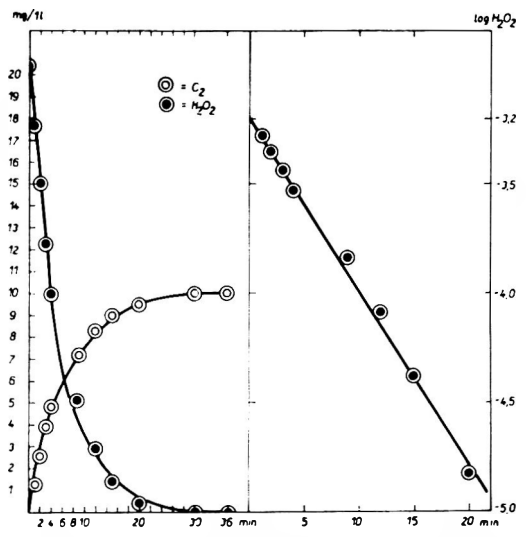

Fig. 4. Graphic representation of the dependence of oxygen concentration, hydrogen peroxide and logarithm of hydrogen peroxide concentration on the time, under action of glucose oxidase on hydrogen peroxide. Preparation DeeO, 1:3000; pH 5.6; T = 23°C.

The reactivity of catalase can be expressed here again either by the half-life period, or by the rate constant.

If the need is to determine the activities of both glucose oxidase and catalase simultaneously, and to determine not only the time necessary for the removal of oxygen but also of that of hydrogen peroxide from the medium, the determination is carried out in such a manner that the height decreases of the oxygen and of the peroxide waves are recorded alternately. This method is shown in Fig. 5. O_2 content is calculated according to the aforementioned equation. Used for calculating the peroxide content is the following equation:

$$P_t = P_n \cdot \frac{C_t - kA_t - c}{C_o - A_o - c}$$

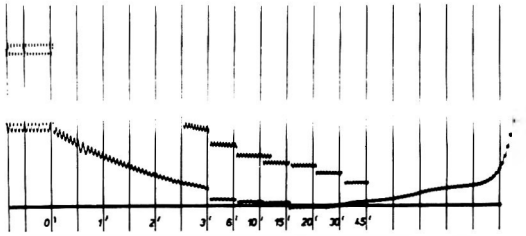

Fig. 5. Limit current of the first and second oxygen reduction waves. Preparation DeeO, 1:5000; pH 5.6; T = 23°C.

P_n = content of hydrogen peroxide corresponding to stoichiometric reduction of oxygen in the air-saturated solution (for 23°C = 9.1 mg/liter)

P_t = hydrogen peroxide in the time t

C_o = height of the double wave limit current at −1.39V/SCE from the zero point of the galvanometer in the time 0

C_t = height of the double wave limit current at −1.39 V/SCE in the time t

A_o = height of the first reduction wave limit current at −0.45 V/SCE in the time $t = 0$

A_t = height of the first reduction wave limit current at −0.45 V/SCE in the time t

c = value of the capacity current at the voltage −1.39 V/SCE

$$k = \frac{C_o - c}{A_o}$$

The value of the capacity current for the measuring apparatus employed and the given condition of determination is measured from the course of the current in a pure electrolyte in the absence of oxygen. In our case, the value of capacity current at the voltage −0.45 V/SCE was zero, at the voltage −1.39 V/SCE and under the given conditions it amounted to 5% of the diffusion current of the second oxygen reduction wave, measured from the zero point of the galvanometer.

(The applied voltage at which the limit current height of the first and second oxygen waves was measured, was chosen to correspond approximately to the middle of the linear part of limit current. The voltage at the chosen points of the two waves has been determined by comparison with the half-wave potential of the thallium wave.)

The constant k applied in the equation ensues from the circumstance that the total height of limit current of the second oxygen reduction wave measured from the galvanometer zero point does not correspond to the double of limit current of the first reduction wave. The constant can be found empirically for the given measuring apparatus and for the conditions of determination. In our case it was $k = 1.85$.

In this simultaneous determination of oxygen and peroxide, the peroxide content cannot be read directly and therefore the values have to be calculated from the graphically represented relationship, which shows the simultaneous course of oxygen decrease and alteration of the peroxide content (see Fig. 6a and 6b). Fig. 6b represents the course

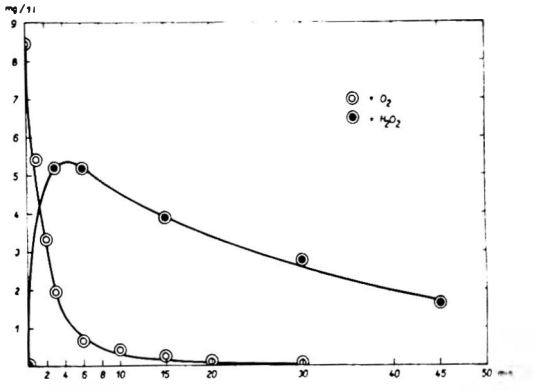

Fig. 6a. Plot of the decrease of oxygen and concentration of peroxide vs. time. Preparation DeeO, 1:1000; pH 5.6; T = 23°C.

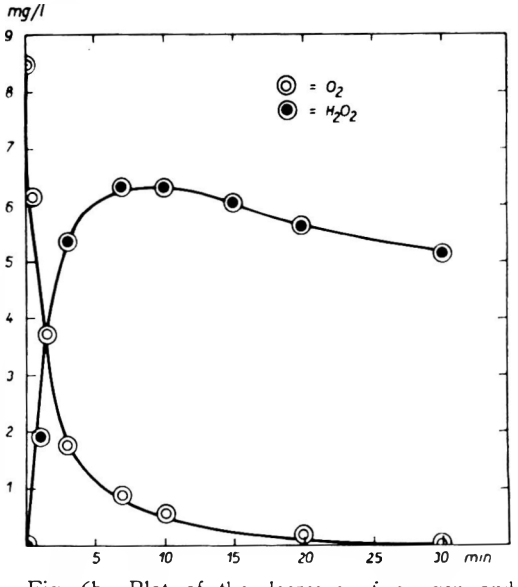

Fig. 6b. Plot of the decrease of oxygen and concentration of peroxide vs. time. Experimental preparation, 1:1000; pH 5.6; T = 23°C.

of the oxygen and peroxide contents in another glucose oxidase preparation, an experimental product of our own. As evident from the graph, this preparation has a noticeably smaller catalase activity than the DeeO glucose oxidase. The activity of both enzymes was also determined with the method

described in various phases of the glucose oxidase production. The method proved to be generally applicable.

Besides the determination under restricted air access, the decrease of enzyme activity during its long-term action at sufficient air access can also be determined polarographically. This decrease of activity can be influenced either by a variation of pH, by increase of peroxide concentration, or by exhaustion of the substrate. The determination is performed as follows: the aeration is interrupted and the oxygen decrease determined at certain intervals, in the same way as in the preceding cases. Yet a sufficient amount of glucose must be added to the reaction medium. Fig. 7 shows the course

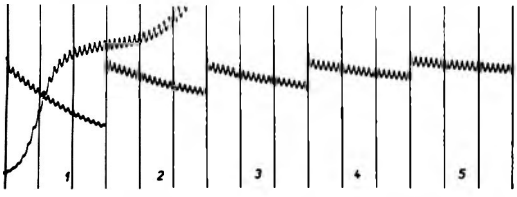

Fig. 7. Limit current of the first oxygen reduction wave during long-term action of glucose oxidase under oxygen excess: 1) immediately after addition of glucose oxidase; 2) after 30 min; 3) after 60 min; 4) after 90 min; 5) after 120 min. Preparation DeeO, 1:4000; pH 3.6; T = 23°C.

of glucose oxidase activity decrease during a longer action of the enzyme at excess of oxygen. The preparation DeeO was used in a dilution of 1:4000 at pH 3.6 and 23°C. The single determinations were made immediately after addition of the enzyme, and then after 30, 60, 90, and 120 min. Each measurement took 1.5 min, interrupting aeration of the solution.

It should be added that it was clear to us during the above-described polarographic determinations of enzyme action that certain factors can distort the results obtained: This is the influence of catalase on the oxygen curve shown in the two-stage reduction by augmentation of the first, and diminution of the second wave (Koutecky *et al.*, 1953). To clear up this question, we arranged a separate experiment. A buffer solution was saturated with oxygen in the absence of glucose, and the two oxygen reduction waves were recorded. The determination was then repeated in the same manner in the presence of the mixed enzyme. The observations were made at the same conditions as in the proper experiments, i.e. at constant temperature, pH, and dilution of the enzyme. Besides this, an additional experiment was undertaken under the same conditions as before, yet with the enzyme 50 times as concentrated. In the first case, a curve of a two-stage reduction was obtained wherein the relation of the two heights of wave was constant before and after addition of the enzyme. In the second case the height of the first reduction wave was increased by 10%, and the peroxide wave decreased by the same value. It is then evident that the enzyme, in the concentrations used in our experiments, did not influence the limit current height of the oxygen reduction wave, which was used to follow the activity.

In conclusion, it appears necessary to summarize the experience obtained and to evaluate on its basis the method of polarographic determinations of the enzyme activity, as follows:

1) The polarographic method is much more rapid.
2) It is suitable for kinetic studies of enzymatic reactions, making it possible to follow said reactions even for short time intervals (below 1 min). Sack *et al.* prefer this method to Warburg's in examining the activity of ascorbate oxidase.
3) The conditions of determination, i.e. the restricted access of oxygen, approximate as much as possible the real conditions under which glucose oxidase is used in practice.
4) The activity decrease can be determined even in long-term enzyme action under oxygen excess.
5) The polarographic method allows determination of the following values:
   a) Evaluation of the total activity of a mixed enzyme.
   b) Determination of the time necessary for complete removal of oxygen from biological materials.
   c) A picture of catalase activity and hydrogen peroxide concentration in the reaction media, thus allowing determination of the time necessary for complete removal of the peroxide.

6) The method permits continuous control of the enzyme action at any desired time interval.

ACKNOWLEDGMENT

The authors feel much indebted to Mr. L. A. Underkofler for supplying them with the preparation DeeO, with which the major part of the experimental work was carried out.

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Enzymatic Studies of Bruised Poultry Tissue[a]

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(Manuscript received October 29, 1963)

SUMMARY

Acid phosphatase activity increased and alkaline phosphatase activity decreased in experimentally inflicted poultry bruises. The activities of both enzymes were influenced by such factors as age of bruise, severity of muscular injury, and previous bruise history. Activity of acid phosphatase in bruised areas was maximum 4–6 days post-contusion, being 2–3 times that in normal muscle. Alkaline phosphatase activity often approached 50% that of the control level on the first and second days after trauma. Under ordinary assay conditions, appreciable acid phosphatase was found to be bound in lysosomes whereas no bound activity was demonstrable in normal tissue. When normal muscle was incubated with 1% trypsin prior to homogenation, however, some bound acid phosphatase activity was detected.

Biochemical and physiological changes associated with bruised tissue have been under investigation in our laboratory for the past several years. Other research investigators have studied biochemical alterations following burns (Merezhinskii *et al.*, 1954), changes in level of protein during regeneration (Gjessing *et al.*, 1947; White *et al.*, 1959; and Guschlbauer and Williamson, 1963), alterations in nitrogenous compounds (Engel, 1952), and changes in connective tissue and lean muscle mass during healing of open wounds (Reynolds *et al.*, 1963). One of the striking changes following trauma is an increase in inorganic phosphate and a decrease in adenosinetriphosphate in the injured area (Jordan and Gray, 1955; Hamdy, 1963). Beatty (1945) found inorganic phosphate, lactic acid, and phosphopyruvate to accumulate following trauma, and adenosinetriphosphate, adenosinediphosphate, phosphocreatine and glycogen to diminish.

Hamdy *et al.* (1957a) showed in cattle that swelling and fluid volume were greatest within 2 days post-bruising, and biochemical changes were maximum on or about the fourth or fifth days, whereas in poultry (Hamdy *et al.*, 1961a) the corresponding activities were noted on the second and third days. Hamdy *et al.* (1961a) further established that proteolytic activities, detected in the damaged areas, were influenced by previous bruising, severity, and age of the bruise.

This report summarizes experiments on the effects of bruising on acid and alkaline phosphatases. Since acid phosphatase has been reported as one of the enzymes bound within the cytoplasmic lysosomal particles (de Duve, 1955), an attempt was also made to differentiate bound acid phosphatase from the free form of the enzyme in bruised and control muscle tissue. Factors other than bruising which may affect the activities of these enzymes were also examined.

MATERIALS AND METHODS

Bruising and sampling. Chickens were kept in constant-temperature rooms (70°F) for at least 5 days prior to bruising. Unless otherwise indicated, bruises were inflicted on the pectoralis major muscle, using 3 blows by the standard technique previously described by Hamdy *et al.* (1961a). At definite intervals after bruising, bruised and control chickens were sacrificed by decapitation and tissues were immediately excised, wrapped in Cryovac packaging film, immersed in ice, and used for analysis within 1–3 hr.

Enzyme assays. *Total acid phosphatase.* The assay system for acid phosphatases, a modification of that used by Valentine *et al.* (1961), contained 0.5 ml of 20% tissue homogenate (prepared in distilled water in a Servall Omni-mixer) and

[a] Approved as journal paper 331, College Experiment Station, College of Agriculture, University of Georgia, Athens, Georgia.

Supported by grant (EF-00164) from the Nat. Inst. of Health.

Presented at the 23rd Annual Meeting of the Institute of Food Technologists, Detroit, Michigan, May 26–30, 1963.

0.5 ml of 0.0125*M* disodium-*o*-nitrophenylphosphate in 0.1*M* sodium acetate buffer, pH 5.0. Enzyme reactions were stopped with 5.0 ml of 2% phosphotungistic acid in 0.1*N* HCl. After centrifugation, the absorbance of 1 ml of supernate in 10 ml of 0.66*N* NaOH was measured at 410 mμ in a Bausch & Lomb "Spectronic 20." This was corrected for the substrate and tissue control absorbances and compared to a standard curve for *p*-nitrophenol. Specific activities were expressed as millimicromoles of nitrophenol liberated per mg protein per 10 minutes of incubation at 37°C.

Free and bound acid phosphatase. To determine free, lysosomal-bound, and total activities, homogenates were prepared in 0.25*M* sucrose containing 1m*M* versenate. Free activities (not enclosed in lysosome particles) were measured directly in the homogenates, as described, but maintaining the reaction mixture in 0.25*M* sucrose. Total activities were measured in a like manner in the presence of 0.2% Triton X-100 (Wattiaux and de Duve, 1956). Bound activity was calculated by subtracting the free from the total activity.

Alkaline phosphatase. The alkaline phosphatase assay was similar to that used for acid phosphatase, except the reaction mixture contained 1.0 ml of 0.1*M* veronal buffer (pH 9.0), 0.5 ml of 0.0125*M* disodium-*o*-nitrophenylphosphate and 0.5 ml of 20% homogenate. Protein concentrations in tissue homogenates were estimated with biuret reagent (Gornall *et al.*, 1949) and crystalline ovalbumin (Nutritional Biochemical Co.) as the standard.

RESULTS AND DISCUSSION

Activity and age of bruise. Twenty-four White Leghorn cockerels, 16–18 weeks old, were bruised. Four unbruised chickens were controls. Samples excised from bruised chickens included the discolored area (bruise), the periphery (not obviously discolored area immediately surrounding the bruise), and symmetrically located control muscle from the unbruised breast. The results (Fig. 1) reveal that acid phosphatase activity was greatest in the bruise, and elevated but slightly less in the periphery. Enzyme action in symmetrically located muscle from the unbruised breast was similar to that in tissues excised from unbruised chickens. Activity in the bruise had definitely increased by the second day and reached a maximum, approximately twice that of control activities, by the fourth day. Healing was morphologically complete on the sixth day. The elevated acid phosphatase may be due to infiltration of leucocytes and macrophages into the injured tissue (Cohn and Hirsch, 1960; and de Duve (1959), active synthesis of the enzyme at the site of injury induced by waste or degradation products from dead cells, as suggested by Tappel *et al.* (1962), removal of some enzyme inhibitor, or a response to peripheral hormonal variations incited by the injury (Young and Gray, 1956; Gray and Beetham, 1957).

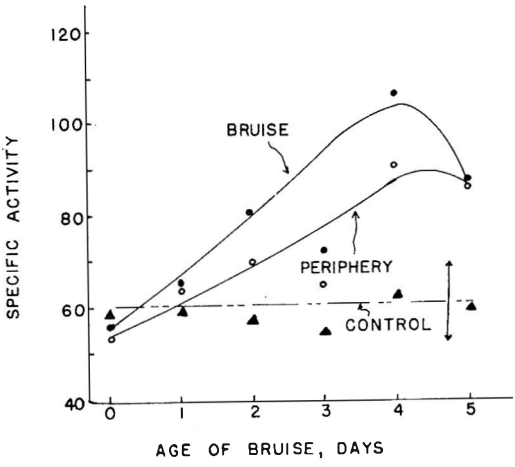

Fig. 1. Effect of age of bruise on acid phosphatase activity in the bruise, periphery, and symmetrically located control muscle. Vertical arrow through average control level denotes standard deviation of the control activities.

Effects of severity of bruising. After the detection of acid phosphatase in bruised tissue, it was decided to examine factors other than age of bruise which might affect acid and alkaline phosphatase activities. Accordingly, 18 chickens were bruised with 2 blows, 18 with 4 blows, and 18 with 6 blows. Birds from each group were sacrificed at various intervals, and bruises were assayed for acid phosphatase activity. A similar experiment was conducted with alkaline phosphatase using three groups of 35 chickens each. The first group was bruised with 1 blow, the second with 3, and the third with 5 blows.

The results (Fig. 2) showed that the timing and degree of maximum activity were directly related to the number of blows applied. Maximum activity occurred on the fourth day in a superficial bruise (2 blows), on the fifth day in a medium bruise (4 blows) and on the seventh day in a severe bruise (6 blows). These represented 1.7-, 2.3-, and 3-fold increases over the control

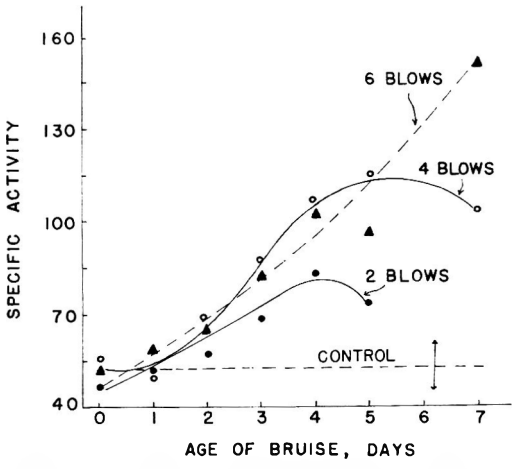

Fig. 2. Effect of age and severity of bruising on acid phosphatase activity in the tissue. Vertical arrow through control activity shows standard deviation.

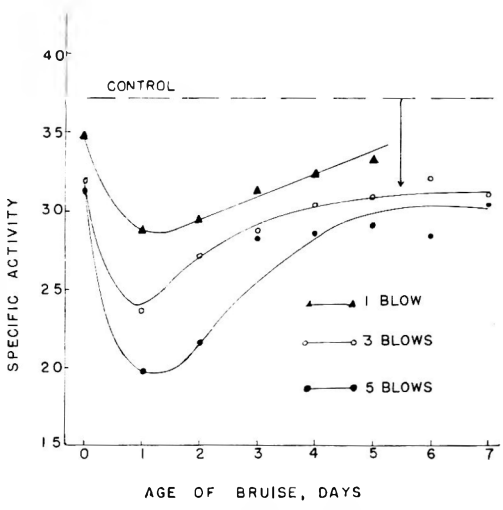

Fig. 3. Effect of age and severity of bruising on alkaline phosphatase activity in the tissue. Arrow extending downward from control level denotes lower limit of the standard deviation.

level for the superficial, medium, and severe bruises, respectively.

These results were similar to those established by Hamdy *et al.* (1961b) for proteolytic activity measured at pH 8.0, in that increasing severity of injury prolonged the duration of enzyme activity, for example, 2–4 days for the proteinase, and 3–7 days for acid phosphatase. In the present study, increasing the number of blows elicited much higher maximum activity, which was not observed with the proteolytic enzyme.

The specific activities of alkaline phosphatase showed a definite decrease within 24 hr post bruising (Fig. 3), and the more severe the bruise the greater was this decrease. Forty-eight hours post-bruising the enzymatic response to severity of contusion presented a complex picture. However, in general, there was a gradual increase from the second to the fourth day to approach the control level. No explanation for these alterations can be given at the present time.

Decreased alkaline phosphatase activity in sebaceous glands and spleen of X-irradiated guinea pigs was reported by Kurban *et al.* (1962) and Ellinger and Stricke (1961) and following burns (Chandrai *et al.*, 1961).

On the other hand, histochemical studies of burns and mechanical injuries have associated high enzyme activities with areas of necrosis, regenerating connective tissue, and leucocytes (Shimizu and Hamuro, 1958; Carranza and Cabrini, 1963; Breedis *et al.*, 1943; Russel *et al.*, 1944; Fell and Danielli, 1943; McDuffie and Schmittle, 1963).

Using β-glycerophosphate as a substrate and the histochemical technique of Gomori (1952), McDuffie and Schmittle (1963) reported little alkaline phosphatase activity in normal muscle and greatly accelerated activity 48–72 hr post-bruise. It is interesting to note that the present investigation on tissue homogenates showed higher activity in control tissue than in bruised tissue. This could be a reflection of the nonspecificity of *o*-nitrophenylphosphate as compared to β-glycerophosphate. Alternatively, the absence of histochemically demonstrated enzyme in normal muscle may be a result of loss of the enzyme during preparation of the slide for histochemical examination, denaturation of the enzyme, or inaccessibility of the normal enzyme to the substrate. It is also possible that the histochemically manifested enzyme may be newly synthesized material, enzyme already present in the tissue and merely made available to the substrate by the action of the autolytic enzymes, or, possibly, it could be from a source external to the bruised area itself, for example, plasma or white blood cells. Novikoff (1959) stated that most histochemical demonstrations of enzymatic functions are not quantitative but that their ultimate value is in locating the sites of enzyme activity in tissues and cells.

Effect of repeated bruising. Twenty-four chickens (group A) were bruised 4 successive times at 8-day intervals, alternating breasts and contusing different areas of the breast. When the fourth bruise was inflicted, a similar number of previously unbruised chickens (group B) were also contused. Three unbruised chickens were controls. The results are shown in Fig. 4 (acid phosphatase) and Fig. 5 (alkaline phosphatase).

Although at all times the acid phosphatase content of bruises from group A exceeded that of bruises from group B, it was only after the third day that these differences showed any great divergence. Group B had an average specific activity of 75.4 from the third to the sixth day with a maximum of 91.6 occurring on the fourth day, compared with an average of 102.5 over the same period and a maximum specific activity of 128.1 occurring on the fifth day for Group A.

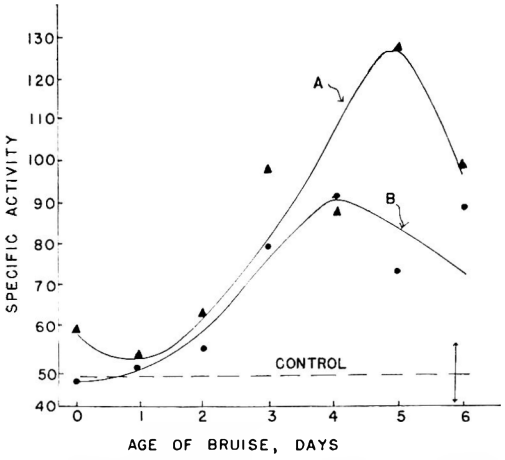

Fig. 4. Effect of age of bruise and repeated bruising on acid phosphatase activity in the bruise. Vertical arrow through control activity denotes standard deviation of controls.

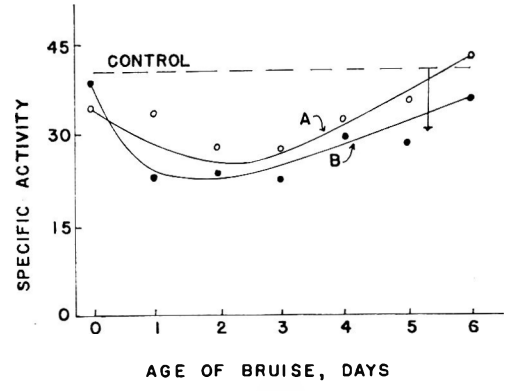

Fig. 5. Effect of age of bruise and repeated bruising on alkaline phosphatase activity in the bruise. Arrow extending down from control level denotes lower limit of the control standard deviation.

Hamdy *et al.* (1961b) demonstrated changes in proteolytic activity as a result of severity and previous bruise history. Again, Hamdy *et al.* (1957a, 1957b) reported that when animals were bruised repeatedly each successive bruise healed more rapidly than the previous one and that bilirubin was detected at an earlier time. In the present investigation, acid phosphatase actively increased with severity of the bruise, and the occurrence of maximum activity was delayed. On the other hand, when 2 groups of chickens (Fig. 4), one previously unbruised (group B) and the other repeatedly bruised (group A), were bruised in a similar manner, the birds experiencing prior bruises seemed able to bring the hydrolytic enzyme into function more rapidly and to a greater extent than those receiving a primary bruise.

Some differences were noted in alkaline phosphatase activity in birds repeatedly bruised and those bruised once, respectively groups A and B (Fig. 5). Group B had lower specific activities during the entire period following bruising and during healing. Whether this is indicative of decreased tissue destruction (less necrosis) in the repeatedly bruised chickens or a more rapid rate of recovery from other factors causing the decreased enzymatic activity is not definitely known. However, previous work of Hamdy *et al.* (1957a, b) supports the latter concept.

Differentiation between bound and free enzyme. The results obtained for free and detergent activated (bound) lysosomal enzymes (Table 1) revealed no bound acid phosphatase in normal tissue with increases in both bound and total phosphatase for 2–5 days after bruising. Although total activity increased with age of bruise from 2 to 5 days, the percentage of bound acid phosphatase remained relatively constant.

Low yields of bound lysosomal enzymes in certain animal tissues have been related to their resistance to homogenization (de Duve, 1959). The greatest amount of bound acid phosphatase in bruised tissue (Table

Table 1. Total acid phosphatase activity and percentage of bound acid phosphatase activity in normal and bruised muscle.

Age of bruise (days)	Total activity	Percentage of bound activity
Unbruised control	34	0
0	34	0
1	44	14
2	93	24
3	135	29
4	117	28
5	143	28

Activity = millimicromoles of nitrophenol liberated per mg protein per 10 min of incubation at 37°C.

1) was found at the same stage of healing (2–3 days post-bruise) where Hamdy *et al.* (1961b) detected maximum proteolytic activity. These considerations, along with the well recognized labile nature of the lysosomal membrane, led the present authors to the following question: would the increased bound acid phosphatase measured in bruised tissue be due to an actual elevation of bound enzyme content in the injured area or would it be due to a decreased resistance of the tissue to homogenization brought about, at least in part, by proteolysis? To elucidate the question, normal pectoralis major muscle was minced to fragments 2–4 mm in diameter and incubated at 37°C for various periods in 0.25*M* sucrose containing 1% trypsin powder. Excess trypsin was removed by repeatedly washing the tissue with sucrose solution following incubation, and the trypsin-treated muscle fragments were homogenized and assayed for free and total activities as described. The results (Table 2) showed that normal muscle does contain some bound acid phosphatase. However,

Table 2. Increase in bound acid phosphatase in normal tissue following treatment of muscle fragments with 1% trypsin.

Incubation (min)	Percentage of bound acid phosphatase activity	
	Expt. 1	Expt. 2
0	0	0
2	2	[a]
5	5	9
10	14	9
20	11	13
40	10	11

[a] Activity not measured.

the measured bound fraction was less than that detected in 3-day-old bruised tissue.

The significance of these findings is a confirmation of the labile character of lysosomal membrane integrity, and thus brings forth the question as to the validity of interpretations of data concerning differences of bound and free enzyme activities in healthy and injured tissues. This is especially true where tissues are difficult to homogenize or in instances where there are obvious physical or textural differences between normal and abnormal tissues.

While Tappel *et al.* (1962) reported 28% of acid ribonuclease, 57% of cathepsin, and 57% of β-glucuronidase to be bound in normal chicken muscle, no detectable amount of bound acid phosphatase was found in the present study in the absence of trypsin treatment prior to homogenization. However, the aforementioned author did not report on bound acid phosphatase in normal fowl muscle.

ACKNOWLEDGMENTS

The authors express their appreciation to Dr. K. N. May, University of Georgia Poultry Department, and Dr. S. C. Schmittle for help in behalf of this project. We are also indebted to the Bacteriology Department, University of Georgia, for the use of its animal rooms.

Triton X-100 was donated by Rohm and Haas, Philadelphia 5, Pennsylvania.

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Relationships of Free and Bound Water to Subjective Scores for Juiciness and Softness and to Changes in Weight and Dimensions of Steaks from Two Beef Muscles During Cooking[a]

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(Manuscript received August 31, 1963)

SUMMARY

Free and bound water were determined by a modified hydraulic-press method in raw and cooked steaks from two beef muscles, longissimus dorsi (LD) and biceps femoris (BF). One-inch steaks from each muscle were cooked to final internal temperatures of either 61, 68, 74, or 80°C. LD and BF had similar percentages of free and bound water in raw steaks and in steaks cooked to either 61 or 68°C. LD contained a higher percentage of free water at 74 and 80°C. Losses of free and bound water and weight were evident at each increase of temperature. Dimensional measurements made on steaks at different temperatures indicated that LD became shorter, wider, and thinner and BF became longer, narrower, and thinner at 61, 68, and 74°C. A large percent of the total change in either free and bound water or in dimensions of steaks occurred between 74 and 80°C. Subjective scores for juiciness and softness indicated drier and harder meat at each increase of temperature. Scores for softness to tongue and cheek were more consistently correlated with free water than any other subjective measure. Possible relationships between the changes brought about by cooking were discussed.

INTRODUCTION

How sensations associated with eating beef are related to water within the muscle is not clearly understood. The water component of meat would seem to influence its flavor, juiciness, and tenderness. Efforts to associate subjective impressions of meat quality and moisture content have not been entirely successful. Several investigators, including Smith (1933), Clark and Weatherby (1937), Satorius and Child (1938), Hall *et al.* (1944), and Gaddis *et al.* (1950), found no relation between subjective scores for juiciness and the amount of expressible juice or fluid. But Tannor *et al.* (1943), Hardy and Noble (1945), and Tilgner and Osinska (1956) reported good correlations between subjective and objective measurements of juiciness. Much of the disagreement may hinge upon different definitions and interpretations of juiciness.

The numerous methods of determining either the water within meat or the water-holding capacity of meat were reviewed by Hamm (1960). The most widely used and most frequently modified method is the hydraulic-pressure technique (Grau and Hamm, 1953; Wierbicki and Deatherage, 1958). This procedure and that of Asselbergs and Whitaker (1961) were modified for studies reported in this paper.

Beef is eaten after heating or cooking. The effect of heat on the water within meat influences the eating quality of the meat. These effects of cooking or heating have been discussed by Hamm (1960), who suggested that the amount of water bound to the tissues rather than the amount of expressible juice may be related to juiciness of meat. Possible relationships of the water in meat to subjective scores for juiciness and softness have

[a] This study was supported in part by Southern Regional Project SM-19, "Quality of Beef and Factors Motivating Beef Purchases."

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been pointed out by Cover *et al.* (1962b). It is within this background of multiple causes of tenderness that the water in meat has been studied.

EXPERIMENTAL

The meat used was obtained from two groups of animals about 16 months old produced at Texas Agrciultural Experiment Station No. 23. Two muscles, longissimus dorsi (LD) and biceps femoris (BF), were removed after the carcasses had aged 7 days at 36°F. Steaks 1-inch thick were cut from each muscle (opposite last thoracic vertebra in LD; dorsal end of BF in round), frozen at −20°F, and stored at 0°F until used.

Raw steaks, and steaks cooked to internal temperatures of 61, 68, 74, and 80°C, were used. The cooking procedures were described in detail by Cover and Hostetler (1960). The outside edges were trimmed from three sides of the steaks prior to cooking. The fat side was left intact during cooking, but was trimmed from all steaks before grinding. The cooked steaks were cut into convenient pieces and finely ground unless otherwise noted. The raw steaks were trimmed, cut, and ground while frozen.

Total water was determined by the method of the AOAC (1955). Bound water was determined by pressing the meat with a Carver Laboratory Press at a specified pressure, allowing the press fluid to be absorbed by filter paper, and determining moisture remaining in the meat by the AOAC method. The term "bound water" as used in this paper indicates water that was not pressed from the meat at a pressure of 12,500 lb. "Free water" refers to water pressed from the meat at this pressure, or is the difference between total and "bound" water.

Preliminary investigations indicated that the pressed meat would adhere to the filter paper and could not be separated completely from it. After several trials the following procedure was adopted: the sample of meat was placed on a piece of aluminum foil smaller than the pressed sample would be; a second piece of foil was placed on top of the sample. The sample enclosed by the two pieces of foil was placed on filter paper and pressed at the specified pressure for one minute. During the pressing, the meat spread into a thin layer extending beyond the foil onto the filter paper; the moisture spread onto and was absorbed by the filter paper. On removal from the press the two pieces of foil, with the thin layer of meat inside, were pulled away from the filter paper, the edges of the foil and fragments of meat extending beyond the foil were trimmed away, the foil coverings were pulled apart, and the pressed meat was scraped into a previously dried and weighed moisture can. The can and meat were weighed to obtain the sample weight; moisture was determined as mentioned above, and was termed "bound water." Total moisture minus the bound water indicated the amount of free or loose water. Duplicate determinations were made for both total and bound water.

The pressure to be applied and the size of sample were determined in a series of experiments summarized in Fig. 1. Pressures ranged from 5000

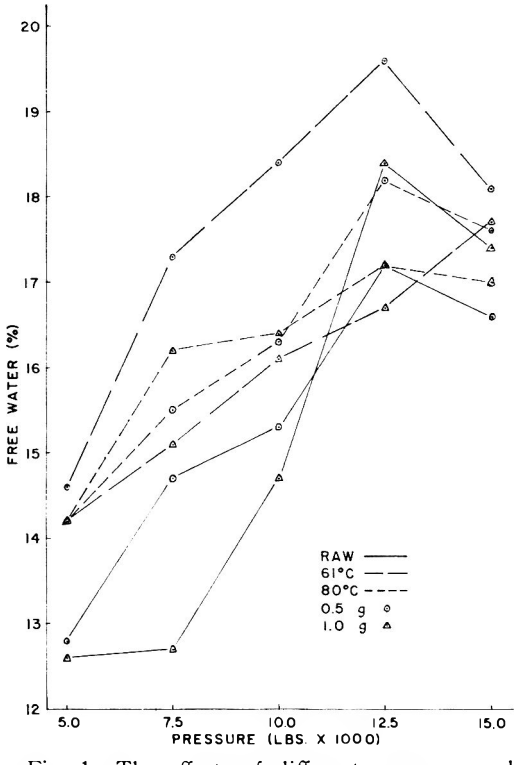

Fig. 1. The effects of different pressures and sample sizes on the amount of free water in raw and cooked steaks.

to 15000 lb as registered on the gauge of the Carver press. Two sample sizes, 0.5 ± 0.1 and 1.0 ± 0.1 g, from raw steaks and steaks cooked to final internal temperatures of 61 and 80°C and which had come from the LD muscle of one animal were used. In each series except one (the treatment at 61°C and sample size of 1.0 g) the maximum amount of free water or the minimum amount of bound water was obtained at a pressure of 12,500 lb. In most cases at the same pressure the 1-g sample had a smaller percentage of free water than the 0.5-g sample. Selection of approximate sample size becomes rather arbitrary since the ratio of free and bound water tends to vary with the amount of sample. In these studies samples weighing about 1 g were pressed at 12,500 lb for the determination of free and bound water.

Data were obtained on raw steaks and steaks cooked to 61 and 80°C from 10 animals of group I; total and bound water were measured after grinding. In 15 animals of group II, steaks were cooked to 61, 68, 74, and 80°C; total moisture was determined on samples after grinding, but bound water was determined on small pieces of meat about the size of a judge's sample (Cover *et al.*, 1962a).

The subjective panel scores for juiciness, softness to tongue and cheek, and softness to tooth pressure were determined as defined by Cover *et al.* (1962a).

Measurements were made of the length, width, and maximum and minimum thickness of steaks before and after cooking from 14 of the 15 animals of group II. The two thickness measurements were averaged to obtain a value for the steak.

RESULTS AND DISCUSSION

Total and bound water were determined in raw and cooked steaks from 10 animals in group I, and in cooked steaks from 15 animals in group II. Table 1 summarizes the percentages of free and bound water in raw and cooked meat from these animals. The LD and BF had similar percentages of free and bound water in raw steaks (group I) and in steaks cooked to either 61 or 68°C (group I and II). When heating was continued to higher temperatures of 74 and 80°C, the LD contained a greater percentage of free water than BF; this is particularly evident at 80°C in group II.

The differences between the two groups of animals in the percentages of free and bound water were probably due to the treatment of samples. Samples from animals in the first group were ground prior to pressing, but small pieces of meat about the size of a judge's sample (see Cover *et al.*, 1962a) were used in the second group. Differences in sample size and the effects of grinding influence the results. In both groups total water was determined on ground samples.

Fig. 2 graphs changes during cooking in the percentages of free and bound water and in the total weight of the steak. Total composition of the steaks was divided into free water, bound water, and solids (or everything except water), each expressed as a percentage of the weight of the uncooked steak. Thus the amounts shown in Fig. 2 for bound and free water in cooked steaks are not the percentages reported in Table 1 for bound and free water in cooked steaks. Weight losses during cooking were considered in the calculations for Fig. 2. These

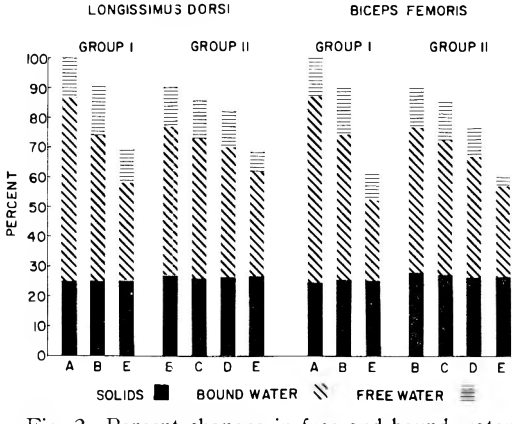

Fig. 2. Percent changes in free and bound water and in solids of steaks from LD and BF during cooking. Treatments indicated by letters were: A, raw; B, 61°C; C, 68°C; D, 74°C; E, 80°C.

Table 1. Percentage of free and bound water in raw and cooked steaks from LD and BF.

	Water content (%)[a]			
	LD		BF	
	Free	Bound	Free	Bound
Group I[b]				
Raw	12.9±1.6	61.5±2.0	12.8±2.1	61.9±1.9
61°	17.3±1.8	54.4±1.1	17.4±2.7	54.0±1.3
80°	15.8±2.9	47.4±1.8	12.2±2.5	45.9±2.0
Group II[b]				
61°	14.4±1.3	55.4±1.4	14.5±2.1	54.2±1.9
68°	14.5±1.4	54.6±1.2	14.4±1.9	53.3±2.0
74°	14.7±1.4	52.6±1.0	13.0±2.2	52.2±1.4
80°	9.7±2.0	51.6±1.0	4.5±2.1	50.7±1.2

[a] Mean and standard deviation.
[b] Group I had 10 animals; Group II had 15 animals.

calculations indicated cooking losses of fat and other solids of less than 2%. These losses were not considered further in the discussion of weight loss.

Weight losses at 61°C were the same for LD and BF. There was an increase in the amount of free water at 61°C in all steaks. The loss of bound water equaled the loss in weight plus the increase in free water. Thus, until the steaks reached 61°C, bound water was converted to free water faster than free water was lost from the steak, resulting in a gain of free water.

Loss of weight proceeded gradually as the temperature increased from 61 to 68 and 74°C in LD. Since the conversion of bound to free water nearly equaled the loss of weight in LD steaks there was little change in the amount of free water present as the temperature increased. Weight loss was slightly greater between 61 and 68°C and much greater between 68 and 74°C in BF than in LD. Since the conversion of bound to free water in BF was not equal to the weight lost at 74°C, there was a slight reduction in the amount of free water present at 68 and 74°C.

Loss in weight, and in bound and free water, was greatest between 74 and 80°C in both LD and BF. In LD the loss of free water between 61 and 68° was 0.6% and between 68 and 74° was 0.3%, but the loss between 74 and 80° was 5.4%. Thus, the loss in free water between 74 and 80° was nine times the loss between 61 and 68° and eighteen times the loss between 68 and 74°C. In BF the loss of free water between 61 and 68° was 0.9% and between 68 and 74° was 2.2%, compared to a loss between 74 and 80° of 7.3%. Thus, the loss in free water between 74 and 80° was eight times the loss between 61 and 68° and three times the loss between 68 and 74°C. The loss of bound water during cooking from 74 to 80° was about twice the loss during cooking from either 61 to 68° or 68 to 74°C.

Only temperature was considered as a factor in the relationships of weight of steaks and free and bound water. Differences between LD and BF were due in part to length of cooking time. The cooking times were nearly alike for both muscles cooked to 61 and 68°C. Steaks from LD required 34 min to reach an internal temperature of 74°C, whereas those from BF required 44 min. In order to reach 80°C, LD required 60 min and BF required 83 min. Both LD and BF steaks were 1 inch thick, but BF steaks were wider and heavier. This may account for the longer cooking times of the BF steaks. The longer times of cooking brought about increased losses of free and bound water at lower temperatures in BF than in LD.

Numerous workers including Hamm (1960) and Cover *et al.* (1962b) have pointed out the differences in free and bound water. In the raw state a certain amount of the total water is bound to the multiple proteins within muscle, and the remainder of the moisture is loose or free; in the animals studied the bound water was about 61% and free water was about 13 or 14% of the total muscle composition. As the meat was heated, bound water was released and became free. Total and free water changed only slightly until above 74°C, where evapo-

Table 2. Mean percentage change in thickness, length, and width from raw steaks as cooked to four temperatures.

	% change in dimensions[a]			
	61°C	68°C	74°C	80°C
LD				
Thickness	−18.7±5.3	−22.8±5.3	−20.1±4.6	−25.2±3.7
Length	− 6.5±3.9	− 8.6±5.2	− 8.3±3.8	−12.9±6.0
Width	10.5±6.3	13.6±7.9	12.3±9.3	15.7±8.4
BF				
Thickness	−18.1±4.0	−20.3±6.4	−23.3±3.1	−25.9±7.1
Length	4.5±2.8	7.5±5.4	7.8±4.6	− 3.5±4.6
Width	− 2.2±5.7	− 1.4±5.2	− 2.7±5.7	− 8.2±6.3

[a] Mean and standard deviation of 14 animals from group II.

ration from the surface of the meat apparently begins to have a larger effect.

The possible relationship of water loss to change in dimensions of steaks as they cooked was investigated. Table 2 gives mean percent changes in the length, width, and thickness of cooked steaks.

Steaks could change dimensions in several ways as they are heated. Increases in over-all length, width, or thickness could be brought about by changes in position of bundles of muscle fibers within the steak. Decreases in length, width, or thickness could be brought about by loss of water from between and from within the fibers, by shortening of the fibers, or by shortening of connective tissue. It is most likely that all of these take place simultaneously as steaks are heated.

Fibers in LD steaks fan out from the approximate center of the fat side of the steak toward the corners and side opposite the fat edge (across the width) and at an angle approximately 45° to the cut surface of the steak. The fibers in BF steaks run in the direction of the length of the steak, parallel with the fat edge and nearly parallel with the cut surface of the steak.

Changes in thickness were similar, but changes in length and width were different for steaks from LD and BF. Both LD and BF became thinner at all temperatures. Much of the decrease in thickness of LD and BF steaks at the lower internal temperatures could be attributed to changes in position of the bundles of fibers. The larger percent decrease at 80°C may be brought about by loss of water and shortening of the fibers.

BF became longer but LD became shorter at 61, 68, and 74°C. Change of position of bundles could account for the increased length of BF. Fibers in BF ran in the direction of the long dimension of the steak. The decrease in length at 80°C was accompanied by a large loss of water. Shortening of muscle fibers also probably took place.

LD probably became shorter because of loss of water from between and within fibers. The fibers of the LD steaks ran more nearly across the short dimension (width) of the steak. The increased shortening at 80°C was also accompanied by a larger loss of water.

Change in width of steaks from the two muscles can be accounted for in a similar manner. LD became wider and BF narrower because of the way the fibers were oriented in the steaks. In LD, change of position of bundles may have exceeded shortening of fibers to maintain increased width at 80°C.

Thus loss of weight as it is associated with loss of bound and free water, and changes in the dimensions of steaks, proceeded gradually to an internal temperature of about 74°C. After 74°C was reached the reactions speeded up, so that a rather large percentage of the total changes in steaks by 80°C occurred between 74 and 80°C.

Fig. 3 gives subjective scores for juiciness, softeness to tongue and cheek, and softness to tooth pressure. Trends of the scores are toward decreasing juiciness and softness, or toward drier and harder meat, with increasing temperatures.

Changes in scores for juiciness follow the general trends of free and bound water. Coefficients of correlation were calculated for each subjective score versus both free and bound water Correlations for juiciness scores and either free or bound water are rather low and are not consistent from temperature to temperature. This, as pointed out above, has been the experience of most other investigators.

Juiciness has been defined for these studies as the amount of juice squeezed out of the meat by a few gentle chews (Cover *et al.* 1962b). Thus, juiciness is the impression of moisture running out of the meat as pressure by the teeth is applied. By definition, this would be the free or loose water influencing this mouth senstaion. This conclusion is not in accord with the suggestion of Hamm (1960) that juiciness is a reflection of the amount of water retained or bound by the meat tissues. Differences may exist because of definitions. If juiciness is scored during the first few chews of the meat, free water would seem to be the major factor. However, if juiciness is scored after prolonged mastication and the general impression of over-all wetness is received, numerous factors such as fat content, flow of

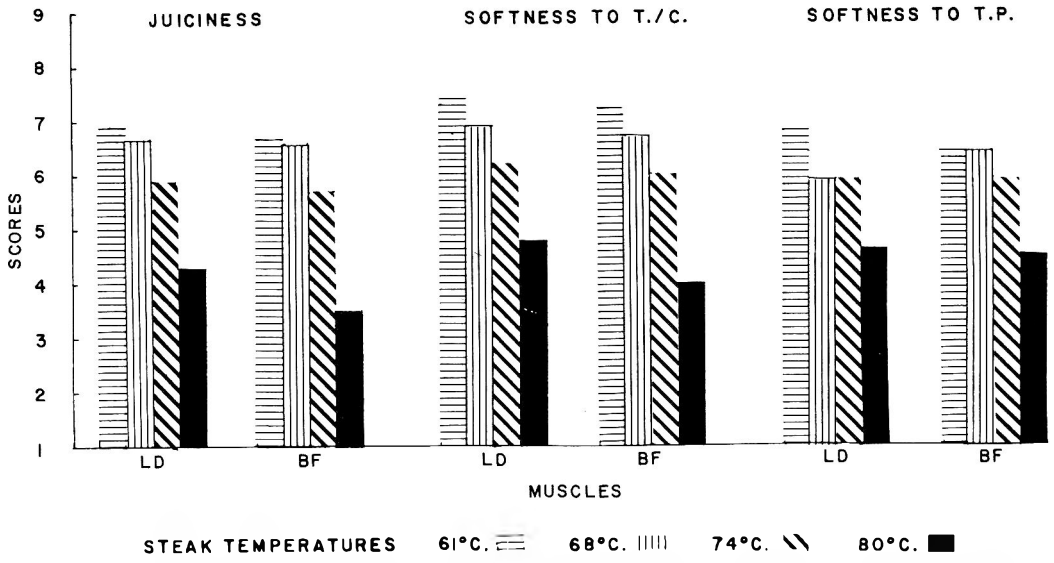

Fig. 3. Average scores for juiciness and softness components in steaks from LD and BF cooked to four temperatures. Subjective scores ranged from 1 (very dry or very hard) to 9 (very juicy or very soft).

saliva, softness, and both free and bound water may enter the picture.

Scores for softness to tongue and cheek were more consistently associated with free water than was any other subjective score.

At the various temperatures, correlation coefficients for LD in animals of group II were 61°C, 0.32; 68°C, 0.24; 74°C, 0.23; and 80°C, 0.52. For corresponding temperatures in BF the coefficients were 0.22, 0.16, 0.57, and 0.73. The coefficients for this panel score and bound water at some temperatures were very low and were not consistent from temperature to temperature. Previous work (Cover *et al.*, 1962b) had indicated that softness to tongue and cheek may be related to juiciness in the over-all juiciness-tenderness relationship. The moisture component within meat has a role in these subjective scores, and free water appears to be involved more than bound water.

Coefficients of correlation for free water and scores for softness to tooth pressure were all negative and quite low in LD, and were all positive in BF. At the four temperatures, coefficients in BF from animals of group II were zero, 0.37, 0.51, and 0.62. The scores for softness to tooth pressure were similar for the two muscles at each temperature, but the influence of water—either free or bound—may be different in the muscles. The tendency of BF to shatter or to become more mealy and the tendency of LD to adhere more tightly at higher temperatures (Cover *et al.*, 1962c) may be involved. At a higher temperature of cooking (100°C as reported by Cover *et al.*, 1962c) the differences in mealiness between LD and BF became larger than in steaks cooked to 80°C. At increased temperatures the apparent adhesion of muscle fibers became greater in LD and less in BF.

The denaturation of muscle protein and effects on hydration of muscle proteins during heating were reviewed by Hamm (1960). As meat is heated, the muscle proteins are denatured and lose their ability to bind either their own water or added water. As a result of these alterations, the eating quality of meat is changed. Data presented in this discussion demonstrate some of these changes during heating and the effects of heating on subjective scores for juiciness and softness components of eating quality. Many relationships are not clear, and only further probing will result in their complete elucidation.

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Chlorophyll Catalysis of Fat Peroxidation[a]

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(Manuscript received November 25, 1963)

SUMMARY

Peroxide apparently occurring in freshly prepared sausage was traced to the catalytic effect of chlorophyll in the sage after fat extraction. The extracts were exposed to ordinary laboratory illumination a day before analysis. No peroxide appeared in extracts of the pork fat alone, nor in extracts of the sage alone. But mixtures of fat extract with sage extract developed peroxide. Extracts of green leafy material from other species and purified chlorophyll likewise developed peroxide with fat extracts. This effect is ascribed to the porphyrin structure. Extracts containing fat and chlorophyll should be kept in the dark until peroxide estimation.

INTRODUCTION

Peroxide numbers (ml 0.002*N* thiosulfate per gram of fat) of a trace to more than three have been found consistently by the authors in freshly made sausage. No peroxide was found in the fat tissue used in the sausage. Samples taken from a sausage mix at each step in its preparation indicated no peroxide until the final mix. Sage was the last ingredient added.

EXPERIMENTAL

An ethylene chloride (EC) extract (Watts and Peng, 1947) of a mixture of sage and pork fat tissue developed a peroxide number (Wheeler, 1932) of 3.2 after standing a day exposed to usual laboratory incandescent illumination, but not near a window. No peroxide developed in individual extracts of the sage or fat under the same circumstances. After standing five days, the mixture extract increased the peroxide to 17.0, but no peroxide had developed in the extract of the fat alone.

When the individual extracts of fat and sage were mixed in equal parts, a peroxide number of 14.3 developed in three days. The same kind of reaction was given by sage from four different sources, including rubbed and leaf sage. All sage extracts were green, and those with the most intense color developed most peroxide with fat. Extracts were made in the proportion of 15 g of fat tissue or 0.3 g of sage to 75 ml EC. EC extracts from dehydrated alfalfa, fresh green leaves (1.5 g/75 ml) of alfalfa, bluegrass, wheat, elm, and lilac, and a solution of 3 mg chlorophyllin (NaKCu) in EC with 1.5 ml acetic acid/75 ml were prepared in dim light and stored in the dark. Duplicate sets were made of mixtures of these individual extracts with equal volumes of fat extract. For 24 hr one set was kept in the dark, and the other in a west window but shielded from direct sunlight.

RESULTS AND DISCUSSION

Only traces of peroxide developed in any of the mixtures kept in the dark. The peroxide developed was roughly 25 times as great in the mixtures containing extracts of fresh green leafy material exposed in the window as in the fat extract alone under the same conditions (Table 1). Difficult solubility of chlorophyllin in EC made it impossible to bring the optical density at 635 mμ above 58% of that of alfalfa extract. This relation is comparable to the relative peroxide activity of the two extracts exposed to light (46%).

An EC solution of malachite green developed no peroxide with fat under window-light exposure. The action of alfalfa extract was not inhibited by malachite green. Thus it seemed that the catalysis of peroxide formation was attached to a specific plant extractive, and not to a direct photochemical effect on the fat due to color absorption in the extract. The activity decreased as the color of the extract was bleached by light.

[a] Contribution no. 45, Department of Biochemistry, no. 297, Department of Animal Husbandry, Kansas Agricultural Experiment Station, Manhattan, Kansas.

Table 1. Effect of extracts of green leafy material of various species on peroxide formation in pork backfat extracts in 24 hr.

Extract in ethylene chloride	Peroxide number[a]	
	In dark	In light
Exposed to interior laboratory illumination		
Fat	0	0
Fat with:		
Sage, rubbed	0	4.7
Alfalfa, dehydrated	0	13.0
Exposed in window, shielded from direct sunlight		
Fat	0	2.9
Fat with:		
Sage, rubbed	0	20.0
Alfalfa, fresh	0.81	84.2
Bluegrass, fresh	0.57	74.6
Wheat, fresh	0.54	86.1
Elm, fresh	0.63	69.0
Lilac, fresh	0.39	77.9
Chlorophyllin (NaKCu)	0	39.0
Malachite green	0	0
Chlorophyll, purified	0.57	80.0

[a] Milliliters of 0.002*N* thiosulfate/g fat.

Chlorophyll was strongly indicated as the active material. This indication was substantiated by an EC solution of chlorophyll from fresh green alfalfa leaves purified by the method of Zscheile (1934) with final adsorption on a sucrose column, washing with petroleum ether, and elution with EC. The eluate was diluted with EC to the optical density shown by the fresh alfalfa extract used in Table 1. These 2 solutions had practically the same effect on peroxide formation, as indicated in Table 1.

This catalytic effect of chlorophyll in the presence of light on peroxide formation in olefinic fats has been observed by others (Taufel *et al.*, 1959; Chipault and Lundberg, 1947). Such references are few and generally remote from the literature on food research. The effect of small quantities of chlorophyll in herbaceous seasonings used in foods could easily be unsuspected and overlooked. This effect is probably due to the porphyrin structure in the chlorophyll molecule. Sharp (1960) used various porphyrin derivatives to convert olefins to hydroperoxides and peroxides under light.

To apply these findings to the problem of peroxide in fresh sausage mix, sausage was prepared under subdued light and divided into two parts. One part was kept in the dark, and the other was spread on a tray and exposed for 4 hr to strong fluorescent light, comparable to usual practice. A sample from each part was extracted with EC under subdued light. Each extract was divided into two parts, one kept in the dark and the other exposed to laboratory illumination for 24 hr, including 4 hr in the window.

Neither extract in the dark showed any peroxide. Both extracts exposed to light gave peroxide values of 17–20.

The conclusion is that the incipient peroxide was developed, not in the sausage itself, but in the extracts exposed to light in the analytical laboratory. The precaution of keeping the extracts in the dark until analyzed is obviously essential.

As long as chlorophyll remained an integral part of the plant structure, it apparently made insufficient contact with fat in the sausage to promote its oxidation when exposed to light. Sage as a whole has been shown (Hall *et al.*, 1962) to behave as an antioxidant in sausage under prolonged frozen storage.

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Detection and Isolation of Multiple Myoglobins from Beef Muscle[a]

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(Manuscript received December 2, 1963)

SUMMARY

At least 3, and probably 4, distinct myoglobins were detected in bovine muscle by their different mobilities on starch-gel electrophoresis. The 3 major components, labeled Mb I_A, Mb I_B, and Mb II and respectively comprising 10–15, 2–5, and 80–88% of the total myoglobin, were separated and isolated on DEAE cellulose columns employing a stepwise development technique. Various purification and separation procedures are discussed.

INTRODUCTION

The myoglobin pigment of a number of animal species has been reported to occur as multiple, electrophoretically-distinct, protein molecules. Rossi-Fanelli, and Antonini (1956), using paper electrophoresis, detected 3 distinct myoglobins in solutions of both crystalline human myoglobin and water extracts from human muscle. Also in 1956, Rossi-Fanelli and Antonini (Rossi-Fanelli *et al.*, 1960) detected 3 myoglobin components in similar preparations from tuna muscle. Rumen (1959) reported the isolation of 5 electrophoretically-distinct myoglobins from seal muscle by chromatography on carboxymethyl (CM) cellulose. Akeson and Theorell (1960), using CM cellulose and diethylaminoethyl (DEAE) cellulose chromatography, isolated 3 myoglobins from horse muscle. Crystalline sperm-whale myoglobin was chromatographically resolved into 5 distinct components by Edmunson and Hirs (1961) on Amberlite IRC-50 ion-exchange resin.

In 1955, Lewis and Schweigert demonstrated the heterogeneity of crystalline bovine myoglobin by free-boundary electrophoresis. Preliminary attempts in this laboratory to purify bovine myoglobin chromatographically also indicated its multiple occurrence. The present study was undertaken to isolate the multiple myoglobins of beef muscle for subsequent physical and chemical characterization studies.

EXPERIMENTAL METHODS

Preparation of crude myoglobin. Cow rounds were boned and trimmed of visible fat. The lean meat was ground and packed into Cryovac bags, which were then evacuated and stored at approximately −29°C. The meat, in 10-lb lots, was thawed at room temperature and homogenized for 30–50 sec in a Waring blender with an aproximately equal weight of water. The thick slurry was frozen at approximately −29°C and then thawed at room temperature. As the slurry thawed, the viscous liquid was filtered through gauze and collected. The volume of liquid was reduced from approximately 7 L to less than 1 L by pervaporation at room temperature in cellulose dialysis tubing. The rate of pervaporation was sufficient to maintain the temperature of the filtrate at 14-16°C.

The gummy precipitate formed during pervaporation was removed by centrifuging for several hours at 2500 rpm and filtering through coarse filter paper. A faster and more complete centrifugal separation was obtained by first heating the pervaporated filtrate for 5 min at 55°C according to the procedure of Snyder and Ayres (1961). No apparent destruction of the myoglobin occurred during the heating process when the pH of the filtrate was first increased to above 6.

The myoglobin-containing filtrate was taken to 65% saturation with solid $(NH_4)_2SO_4$. The precipitate formed was removed by centrifugation

[a] Journal Article 3268, Michigan Agricultural Experiment Station, East Lansing. This study was supported in part by a research grant-in-aid provided by the National Live Stock and Meat Board, 36 S. Wabash Ave., Chicago, Ill. A preliminary report of this investigation was presented at the 21st Annual Meeting of the Institute of Food Technologists, June, 1961, Miami, Florida.

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and filtration, and was then discarded. The filtrate was then completely saturated with $(NH_4)_2SO_4$ and the myoglobin-containing precipitate was collected by centrifugation. The precipitate was dissolved in a small quantity of deionized water and dialyzed at pH 7.0 against a 75% saturated solution (480 g/L) of $(NH_4)_2SO_4$. The resulting precipitate was discarded, and the filtrate, which was designated crude myoglobin, was taken to 90% saturation with solid $(NH_4)_2SO_4$ and stored at approximately −18°C until needed. Centrifugation and $(NH_4)_2SO_4$ fractionation were carried out at 3–4°C, as were all the following electrophoretic and chromatographic procedures.

Sephadex chromatography. The use of Sephadex molecular-sieve chromatography in preparing myoglobin of a purity equivalent to that of the crystalline protein was suggested by Fox (1961). Sephadex G 75 was equilibrated with 0.1*M* triethanolamine (TEA) buffer of pH 7.0, which contained 0.2*M* NaCl, and then packed into 2.5 × 5.5-cm column beds under gravity-flow conditions. The crude myoglobin in 90% $(NH_4)_2SO_4$ was collected by centrifugation and dialyzed against the TEA-NaCl solution until free of sulfate. The myoglobin samples, containing approximately 15 mg/ml were placed on the columns in volumes ranging from 15 to 40 ml and eluted with the TEA-NaCl buffer. The eluate was collected in 10- or 20-ml fractions and read spectrophotometrically at 280 and 525 mμ. Absorbancy at 280 mμ divided by the absorbancy at 525 mμ was used as an index of myoglobin purity of all collected fractions. Fractions having an absorbancy ratio value of less than 4.50 were designated as Sephadex-purified myoglobin and used in subsequent ion-exchange chromatographic experiments.

Electrophoretic detection of multiple myoglobins. Sephadex-purified myoglobin samples were tested for heterogeneity both on Whatman 3-mm paper strips and on Oxoid cellulose acetate strips in a hanging strip, inverted V, Durrum type electrophoretic cell assembly. Veronal and borate buffers at pH 8.6 and at ionic strengths of both 0.050 and 0.075 served as the electrolyte system. Voltage was maintained at 200 volts.

A single moving-boundary electrophoretic experiment was performed. The migration of a Sephadex-purified sample in veronal buffer of pH 8.7 and ionic strength 0.1 and at 1°C was followed for 63.3 min in a Perkin Elmer 38A instrument operating at 10 ma.

The starch-gel electrophoretic technique originated by Smithies (1955) was also tested to demonstrate myoglobin heterogeneity. The gels were prepared by the method outlined by Smithies (1955) using hydrolyzed potato starch (Connaught Medical Research Laboratories, Toronto). The plexiglass electrophoretic cell assembly, featuring 2 removable sections and a slot former, was of the type described by Wake and Baldwin (1961). Heavy chromatographic paper inserted tightly into the ends of the assembly cell prior to filling with the hot, degassed starch, served as electrolyte bridges. The electrodes consisted of 25 cm of platinum wire stretched across the vessels.

The cell containing the cooled gel was placed in position approximately ½ hr before use. The slot former was removed, the myoglobin samples were inserted with a capillary tube, and the entire gel surface was covered with a layer of mineral oil and then with Saran wrap to minimize evaporation. During the 12–14-hr migration, in which the leading zone moved approximately 15 cm, the current decreased from 25 to 10 ma at a constant external applied potential of 200 volts.

Several buffer systems were tested with the starch-gel technique. The borate buffer of Smithies (1955) and a 0.076*M* tris-0.005*M* citric acid buffer at pH 8.9 were each used, both in the preparation of the gel and as the electrolyte. Neither of these buffers, when used alone, gave as satisfactory a separation as the discontinuous buffer system devised by Poulik (1957). By this technique, the gels were made with the tris-citrate buffer but the electrode vessels contained a 0.30*M* boric acid–0.06*M* NaOH buffer.

After electrophoresis the gel was sliced in 3 layers as described by Wake and Baldwin (1961). The center slice was stained with either the protein-detecting dye Amido Black 10 B, as used by Smithies (1955), or the heme-detecting system of Owen *et al.* (1958), which is based on the peroxidase activity of heme groups.

The described starch-gel technique resulted in the satisfactory detection of 3 electrophoretically-distinct myoglobins. The technique was subsequently employed in the routine evaluation of myoglobin chromatographic fractionations.

CM cellulose chromatography. A quantity of CM cellulose containing 0.45 meq acidic groups/g dry weight was prepared by the method of Peterson and Sober (1956) and used in several preliminary experiments. The preliminary experiments were designed to follow as closely as possible the techniques used by Akeson and Theorell (1960) for resolving the components of horse myoglobin.

Samples of Sephadex-purified potassium-ferricyanide-oxidized myoglobin, containing 20–25 mg protein/ml, were chromatographed with 0.02*M* phosphate buffer at pH 6.9. On the basis of spectrophotometric analysis, the eluate was divided into 2 large fractions which were labeled Mb I and Mb II, in the order of emergence from the column. The 2 fractions were then rechro-

matographed on CM cellulose columns in 0.02*M* phosphate buffer at pH 6.4. The 2 fractions, Mb I and Mb II, were also chromatographed at pH 8.5 in 0.01*M* tris buffer according to the technique of Rumen (1959).

Chromatographic experiments were performed on a Whatman CM cellulose (CM-70 containing 0.70 meq acidic groups/g dry weight) column bed of 4.7 × 24 cm to determine the optimum pH and load conditions for resolution of Mb I and Mb II. The pH was varied from 6.82 to 7.20, and the sample, containing 25 mg myoglobin/ml, was applied in volumes ranging from 40 to 100 ml. During the final stages of this study, Mb I and Mb II fractions were routinely prepared by chromatography with 0.01*M* phosphate buffer, pH 6.9, containing 0.01*M* KCN.

DEAE cellulose chromatography. Whatman (DE 50) DEAE cellulose containing 1.0 meq basic groups/g dry weight was equilibrated with 0.02*M* tris buffer and packed into column beds of 2.7 × 50 cm. CM cellulose fractions Mb I and Mb II were each chromatographed on these columns using the buffer at pH 8.3-8.4, according to the technique of Akeson and Theorell (1960), and also both at pH 8.0 and pH 7.8. The fractions obtained by these methods were tested for homogeneity by starch-gel electrophoresis. The chromatography at pH 8.0 was later repeated using 0.001*M* KCN in the equilibrating and developing tris buffer.

Stepwise column development techniques were also tested. Columns of DEAE cellulose equilibrated with 0.02*M* tris buffer at pH 8.6 were prepared. Samples were equilibrated and washed into the columns with the same buffer. It was not necessary to concentrate samples extensively before loading columns equilibrated with 0.02*M* tris at any pH above 8.5. The protein was retained in a single band at the top of the column, while the loading buffer in volumes up to 100 ml flowed through the column. The column was then developed using 4 successive 100-ml volumes of 0.02*M* tris buffer, either adjusted to successively lower pH values (8.30, 8.03, 7.64, and 7.01) or containing increasing molarities of NaCl (0.01, 0.05, 0.10, and 0.20).

The DEAE cellulose chromatographic procedure also involved a stepwise development technique. Since this method resulted in the most homogeneous separation of the 3 electrophoretically-detectable myoglobins, it is described in detail. DEAE cellulose was stirred in several changes of 0.005*M* tris buffer of pH 8.4 until no change in pH was noted. The equilibrated slurry was then added in several portions to the chromatographic tube half-filled with the buffer and was allowed to settle under gravity flow. After each addition, air pressure was applied to compress the bed formed. After air-compressing the last portion of added slurry, several layers of stiff, coarse filter paper (milk filter discs) were placed on top of the column bed, followed by several layers of fine filter paper and then by 2 or 3 more layers of the coarse paper. The filter paper was cut to slightly exceed the column bore, and thus had to be forced onto the column bed. The bed was then further compacted by pressing on the filter paper with a glass cylinder. Columns packed in this manner seldom exhibited channeling. Column beds of 1.7 × 12–15 cm were used to chromatograph protein loads of about 250 mg.

The myoglobin samples (CM cellulose fractions Mb I and II) were equilibrated with the 0.005*M*, pH 8.4, tris buffer by dialysis and washed into the columns with the same buffer. One broad, diffuse myoglobin band was eluted with 0.02*M* tris buffer of pH 8.4. A second, very narrow band was then eluted by applying a buffer mixture containing 4 parts of the 0.02*M* tris buffer and 1 part of the phosphate buffer (0.01*M*, pH 6.9) that was used in CM cellulose chromatography. A third myoglobin fraction was obtained in a very narrow, concentrated band by then eluting with the phosphate buffer alone. The equilibrating and developing buffers all contained 0.001*M* KCN. The pH of the tris-phosphate buffer mixture was 8.3.

RESULTS AND DISCUSSION

Electrophoresis. Neither paper, cellulose acetate zone, nor the moving-boundary electrophoretic experiments demonstrated bovine myoglobin heterogeneity. None of these methods, however, were thoroughly tested in this study. On the other hand, the starch-gel technique demonstrated the heterogeneity of myoglobin on the first attempt. Although the discontinous buffer system of Poulik (1957) proved to be the most sensitive, all buffer systems successfully demonstrated the heterogeneity.

Rossi-Fanelli and Antonini (1958), Rossi-Fanelli *et al.* (1960), Rumen (1959), and Akeson and Theorell (1960) used either paper or free-boundary electrophoresis to test the homogeneity of isolated myoglobin fractions. Present results suggest that the starch-gel technique is more sensitive for this purpose than the other electrophoretic methods. The superior resolving ability of starch gel for many proteins has been well established (Heftmann, 1961).

Initially, the starch gels were made with 7.0*M* urea, as suggested by Wake and Bald-

win (1961), who worked with the casein proteins. Starch-urea gels have several advantages over non-urea gels with respect to handling, storage, and, for some protein systems, resolution. However, it was noted that the light-absorption spectrum of myoglobin in buffered 7.0*M* urea varied markedly from the spectrum of native myoglobin. Hence, the use of urea in starch gels was discontinued. It was also observed that the myoglobin samples, whether only crudely purified or chromatographed on CM cellulose, separated into 1–3 more protein-staining zones on urea gels than on the non-urea gels, but into the same number of heme-staining zones. Each heme-staining zone was also a protein-staining zone. Although the 3 heme-staining zones required a longer (3–4 hr) period to migrate the same distance through the starch-urea gels as through the non-urea gels, they attained the same positions relative to one another in both.

The possibility of myoglobin complexing with borate ions or with metal ion contaminants or of myoglobin aggregation to produce the anomalous results is most unlikely, since it was only the number of non-heme zones that differed in the 2 gel types.

Perkoff *et al.* (1962) implied criticism of earlier work performed on myoglobin heterogeneity by reporting that a large part of the heterogeneity of human myoglobin observed by chromatographic and electrophoretic procedures at alkaline pH values resulted from differences only in the state of the iron of the separated fractions and not from globin structural differences. At alkaline pH values, metmyoglobin exists as a mixture of the acid form and of the alkaline form in which the sixth bond position of the heme iron is occupied by a hydroxyl ion (Lemberg and Legge, 1949). Perkoff *et al.* (1962) eliminated much of the heterogeneity simply by converting metmyoglobin to cyanmetmyoglobin. Prompted by the observations of Perkoff *et al.* (1962), various myoglobin fractions were converted to their cyanide derivatives by addition of 0.001*M* KCN to the dialyzing buffers, and were tested by starch gel electrophoresis. The tris-borate electrolyte system also contained 0.001*M* KCN. The developed cyanmetmyoglobin patterns exhibited no dissimilarities from the routinely-developed metmyoglobin patterns.

Chromatography. Crude myoglobin preparations were partially purified on Sephadex columns. The 280 mμ/525 mμ absorbancy-ratio values of the eluate fractions decreased from the initially high values of 10–15 to values of between 3.9 and 4.5. The initial values were, of course, dependent upon the amount of colorless protein in the preparation. There was no clear-cut separation of proteins on the Sephadex columns, but only one broad, diffuse peak. Also the 280 mμ/525 mμ ratio values did not become constant but fluctuated between 3.9 and 4.5. Myoglobin crystallized by a modification of the method of Lewis and Schweigert (1955) exhibited a ratio value of 4.41. Fox (1961) reported a value of 4.24 for aged myoglobin crystals. The use of Sephadex chromatography in the purification procedure presented no apparent advantage, and was discontinued during the latter stages of this study. Chromatography of the crude myoglobin directly on CM cellulose columns created no difficulty.

A typical elution pattern from a CM cellulose column is presented in Fig. 1. The chromatogram was obtained in a preliminary experiment from a 10-ml myoglobin sample on a small CM cellulose (0.45 meq/g dry weight) column. Chromatography at either pH 6.4 or pH 8.5 did not further resolve fraction I or II. Patterns of the same general shape shown in Fig. 1 were obtained on large columns (4.7 × 25 cm) using Whatman CM cellulose. Sample volumes of up to 80 ml could be chromatographed on

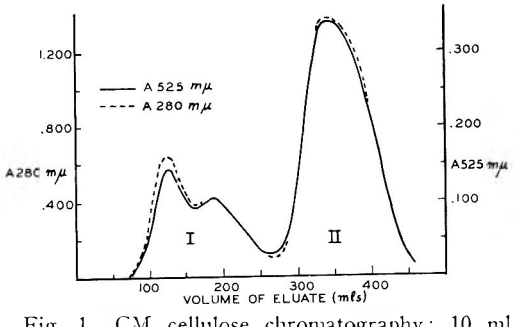

Fig. 1. CM cellulose chromatography; 10 ml sephadex-purified sample containing 20 mg/ml; 0.02*M* phosphate buffer at pH 6.90.

these large columns in 0.02*M* phosphate buffer at pH values ranging from 6.85 to 6.95 without loss of resolution. When 0.001*M* KCN was added to the buffers, the phosphate concentration had to be decreased to 0.01*M* to effect satisfactory development.

Irrespective of the experimental conditions employed during CM cellulose chromatography, neither fractions Mb I nor Mb II was homogeneous. Each contained a mixture of all 3 of the electrophoretically-detectable myoglobins. However, a crude fractionation was obtained. Fraction I was composed largely of the 2 fastest electrophoretically-migrating zones, and fraction II of the slowest migrating zone.

CM cellulose chromatography also served to eliminate most of the protein contamination. Akeson and Theorell (1960) found cytochrome *c* and hemoglobin to be contaminants of $(NH_4)_2SO_4$-fractionated myoglobin solutions but that these proteins remained at the top of the CM cellulose bed while the myoglobin was eluted. The formation of a stationary red band at the top of the bed was also noted in this study. The band was formed regardless of whether or not the myoglobin sample had been previously purified on Sephadex. Attempts to elute this red band failed.

CM cellulose fractions Mb I and Mb II were each resolved into 2 broad, diffuse bands on DEAE cellulose chromatography at pH 8.3–8.4 by the method of Akeson and Theorell (1960). These bands were electrophoretically heterogeneous. On chromatography in 0.02*M* tris at either pH 8.0 or 7.8, both CM cellulose Mb I and Mb II were further resolved. Mb I separated into 6, and Mb II into 4, distinctly visible bands. Again, none of the bands proved homogeneous. The use of 0.001*M* KCN in the chromatographic procedure resulted in the disappearance of several of the bands, thereby confirming the conclusion of Perkoff *et al.* (1962) that myoglobins, differing only in the state of the iron, could be resolved chromatographically.

Stepwise elution techniques, employing buffers of different pH values or NaCl concentrations, did not improve the resolution over the single buffer elution methods. The stepwise procedure utilizing both tris and phosphate buffers, however, resulted in the most homogeneous chromatographic resolution of the 3 electrophoretically-detectable myoglobins attained in this study. The superior resolution obtained appears to involve a displacement effect caused by the phosphate ion rather than a true elution process. The first band eluted was labeled Mb II because it occurred in highest proportion in the CM cellulose fraction Mb II. Similarly, the second and third bands were labeled Mb I_A and Mb I_B in the order of emergence from the column.

The electrophoretic patterns of various myoglobin fractions are exhibited in the accompanying photograph (Fig. 2). The protein migrated from the slots, seen at the right of the photograph, to the positions indicated by the stained zones at the left. Fig. 2A demonstrates the 2 staining techniques used in this study. Patterns 1 to 6 were all formed on the same gel. The gel slice was divided in half prior to staining. The half pictured at the top of Fig. 2A, containing patterns 1, 2, 3, was stained with the protein dye. The other half, containing patterns 4, 5, and 6, was stained by the heme-detecting

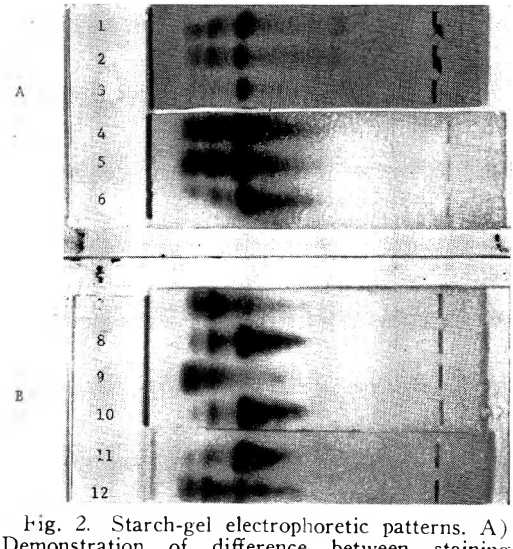

Fig. 2. Starch-gel electrophoretic patterns. A) Demonstration of difference between staining methods. Patterns 1, 2, and 3 were stained with protein dye, and patterns 4, 5, and 6 with heme-detecting method. B) Patterns of myoglobin at various stages of chromatographic resolution. Patterns 7, 9, and 10, respectively, represent DEAE cellulose fractions Mb I_A, Mb I_B, and Mb II. Patterns 8 and 11 were formed from CM cellulose fraction Mb II, while 12 represents CM cellulose fraction Mb I.

method. The photograph illustrates that the protein-staining method caused gel shrinkage and left a dark background. Patterns 1, 2, 4, and 5 were formed from aliquots of the same crude $(NH_4)_2SO_4$-purified myoglobin preparation. Patterns 3 and 6 were formed from aliquots of the same sample of CM cellulose fraction Mb II. Comparing the 2 gel halves, it is evident that the heme-staining method is much more sensitive for the detection of myoglobins.

Fig. 2B pictures myoglobin fractions at various stages of chromatographic separation. All 2B patterns were stained by the heme-detecting method. Patterns 8 and 11 were both formed from CM cellulose fraction Mb II. Pattern 12 represents the smaller fraction, Mb I, obtained from CM cellulose columns. Patterns 7, 9, and 10 respectively represent the DEAE cellulose fractions Mb I_A, Mb I_B, and Mb II. While it is apparent that fractions Mb I_A, Mb I_B, and Mb II are not homogeneous, it is believed that they are not as heterogeneous as the photograph would indicate. The heme-staining technique overestimates, to the naked eye, the relative proportion of minor components. This point is illustrated in Fig. 2A.

Pattern 12 indicates that Mb II, represented electrophoretically as the heavy, slowest-migrating zone in most of the patterns, is composed of at least 2 distinct myoglobins. These myoglobins were not chromatographically resolved. Thus, there would appear to be at least 4 electrophoretically-distinct myoglobins in bovine muscle. It is estimated from observations on both electrophoretic and chromatographic analyses that the 3 myoglobins, Mb I_A, Mb I_B, and Mb II, occur in the relative proportions of 10–15, 2–5, and 80–88%, respectively.

The possibility that the 3 myoglobins are artifacts of the isolation techniques must be considered. The fact that different myoglobin samples — whether prepared by $(NH_4)_2SO_4$ fractionation alone or by $(NH_4)_2SO_4$ fractionation combined with heat treatment, Sephadex chromatography, or crystallization—all produced the same visual results on starch gel electrophoresis, suggests that the myoglobins are naturally-occurring. The myoglobins are separated on cellulose columns in the order expected from electrophoretic analysis. Also, the myoglobins exhibit the same electrophoretic mobility before and after chromatography. Thus, if the myoglobins are denatured during chromatography, they would also have to be denatured in the same way during electrophoresis.

As previously mentioned, myoglobin is not noticeably affected by heat treatment (55°C for 5 min). Various authors (Rumen, 1959; Akeson and Theorell, 1960) have noticed that myoglobin can be split by acid-acetone treatment into heme and globin portions, which can then be recombined to yield products having properties identical to those of native myoglobin. Either of these treatments would seem to be more drastic than the relatively mild conditions encountered during the preparation of Mb I_A, Mb I_B, and Mb II. Since the multiple occurrence of myoglobins has been reported for other animal species, and in one instance (Akeson and Theorell, 1960) a primary structural difference demonstrated, it would seem reasonable to expect the multiple occurrence also in bovine muscle.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical advice of Dr. P. Markakis on paper electrophoresis and of Dr. V. Harwalker on starch gel electrophoresis. Moving-boundary electrophoresis was kindly performed by H. Swaisgood.

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Characterization Studies of Three Myoglobin Fractions from Bovine Muscle[a]

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SUMMARY

Three electrophoretically and chromatographically distinct myoglobin fractions from beef muscle were compared as to absorption spectra, relative heme contents, susceptibility to acid cleavage of heme groups, and autoxidation rates. All 3 myoglobin fractions were found to have identical wavelength positions for minimum and maximum light absorption and to exhibit the same autoxidation rates. Different light absorptivity values and susceptibilities to acid cleavage for the 3 different myoglobins led to the tentative conclusion that structural variations existed at the porphyrin-globin linkages.

INTRODUCTION

Multiple-occurring myoglobins have been isolated from a number of animal species by zone electrophoretic or ion-exchange column chromatographic resolution. Various characterization studies have indicated that the myoglobins within a species have similar properties. Rossi-Fanelli and Antonini (1958) demonstrated the close similarity of 2 myoglobins isolated from human muscle both as to the light absorption spectra of their respective reduced, oxy-, carboxy-, and met-forms and as to their oxygen equilibrium properties. The 2 myoglobins presented oxygen dissociation curves at different temperatures which were exactly alike in hyperbolic shape, high affinity for oxygen, absence of the Bohr effect, and constancy with varying ionic strength and myoglobin concentration. Rossi-Fanelli and Antonini (1960) also reported the 3 myoglobin fractions isolated from tuna muscle to possess identical absorption spectra. Five myoglobins isolated from seal muscle were reported by Rumen (1959) to have the same molecular weight and the same N-terminal amino acid. Although spectrophotometric analysis of the met- and carboxy-derivatives of the 5 myoglobins demonstrated differences in absorptivity in several spectral regions, maximum and minimum absorbances occurred at identical wavelengths.

Akeson and Theorell (1960) isolated 3 myoglobins (Mb I, Mb II_1, and Mb II_2) from horse muscle and found them to have identical iron and sulfur contents. No significant difference was found in the amino acid composition or in the amide nitrogen contents of the 3 myoglobins. The absorption spectra for both the carboxy- and met-derivatives were identical over the range 380–1000 mμ and showed only slight differences in the region of 235–380 mμ. The authors did, however, find a primary structural difference among the 3 myoglobins. Tryptic digestion revealed the presence of 2 specific peptides, both missing in Mb II_1 and present in MbII_2, while Mb I contained one of them. Edmunson and Hirs (1961) performed amino acid analyses on 4 myoglobin fractions obtained from sperm-whale muscle and found no variation among the fractions that would explain their different chromatographic and electrophoretic behaviors.

This report gives a partial characterization of the 3 myoglobin fractions Mb I_A, Mb I_B, and Mb II, isolated in this laboratory (Quinn *et al.*, 1964) from beef muscle.

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[a] Journal Article 3274, Michigan Agricultural Experiment Station, East Lansing. This study was supported in part by a research grant-in-aid provided by the National Live Stock and Meat Board, 36 S. Wabash Ave., Chicago, Ill. It reports a portion of a Ph.D. thesis of the senior author to the School for Advanced Graduate Studies at Michigan State University.

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EXPERIMENTAL

Three myoglobin fractions obtained by DEAE cellulose chromatography of their cyanmet-derivatives (Quinn *et al.*, 1964) were dialyzed for 2 days against several changes of 0.6*M* phosphate buffer of pH 5.8 to dissociate the cyanide. Prior to characterization analysis, the fractions were concentrated by pervaporation and examined spectrophotometrically from 500 to 640 mμ to ensure that no cyanmetmyoglobin was present.

Absorption spectra. Mb I_A, Mb I_B, and Mb II were dialyzed against 0.05*M* phosphate buffer of pH 6.90 and read spectrophotometrically at wavelengths between 725 and 214 mμ. In order to obtain readings below 450 mμ, it was necessary to dilute the samples to approximately 1/3 of their initial concentration.

The carboxy-derivatives of the 3 myoglobins were prepared by a modification of the method of Poel (1949). The deaminizing vessel from a Van Slyke amino nitrogen apparatus was attached to a vacuum line at the top and a source of carbon monoxide (CO) at the bottom. The sample was placed in the reaction chamber and the complete system was evacuated. CO was bubbled slowly through the solution for 5 min. When the pressure within the cell reached atmospheric, as judged by the height of the solution in the connecting side arm to the reagent funnel, the stopcock to the reagent funnel was opened and a small amount of sodium dithionite in a known volume of the 0.05*M* phosphate buffer was added. The system was again completely evacuated and CO was bubbled through the solution for another 5–7 min. The carboxymyoglobin was then allowed to flow into a cuvette which was quickly sealed with paraffin paper. The sample was analyzed immediately in a Beckman model DU spectrophotometer at wavelength intervals from 650 to 400 mμ. After the spectrum was scanned the sample was again read at several wavelengths in the region of 550–630 mμ to ensure that no autoxidation had occurred.

The absorbancy values of both the met- and carboxy-derivatives were converted to their respective molar obsorptivity (E) values by assuming a molecular weight of 17,000 for each of the myoglobins. Protein concentrations were routinely determined by the spectrophotometric method of Waddell (1956) which is based on the far ultraviolet absorption of peptide linkages. The method was tested for accuracy on samples of Mb I_A and Mb II.

Heme content. For determination of heme content the procedure of Keilin and Hartree (1951) was used with minor modifications necessitated by the small amount of Mb I_B sample available. Sample volumes of 1.0–1.5 ml containing a known amount of protein were placed in a test tube. Pyridine, NaOH solution, and water were added to make a final volume of 2.5 ml and the final molarities of pyridine and NaOH 2.1 and 0.075, respectively. A few crystals of dithionite were then added and the contents were thoroughly shaken and allowed to stand for ½ hr. Absorbancy was measured at 557 mμ and divided by the known concentration of protein. Ratio values obtained for the 3 myoglobins in this manner were compared. Because the relative rather than the absolute heme values were of interest, no pyridine hemochromogen standard curve was prepared.

Acid cleavage of hemes. According to Lewis (1954), who originated the method, acid cleavage data make possible the detection of differences in heme-protein linkages. The procedure of Lewis (1954) was modified to permit analysis of small amounts of protein. Samples of Mb I_A, Mb I_B, and Mb II were dialyzed against deionized water until free of phosphate. They were adjusted to approximately the same concentration (0.40–0.50 mg/ml) and NaCl was added to make the final ionic strength 0.02.

The desired amount of 0.04*N* HCl was added to a 5-ml beaker. The volume was adjusted to 0.50 ml with 0.02*M* NaCl solution. The beaker was then swirled while 0.80 ml of the myoglobin sample was added. The pH was measured immediately with a Beckman model G pH meter, and the solution was transferred to a 40-ml centrifuge tube. Eight ml of acetone were added and the tube was stoppered, swirled until cloudiness was observed throughout the contents, and centrifuged for 3 min at 2500 rpm. An aliquot of the supernatant was transferred to a cuvette and read spectrophotometrically at 382 mμ. The method was repeated, varying the proportions of 0.02*M* NaCl and 0.04*N* HCl until a pH range of approximately 2 to 6 was covered. The results, in the form of percentage of heme cleavage, were plotted according to the method of Lewis (1954).

Because of the small amount of Mb I_B available, only a few determinations were made with this protein. The procedure was repeated for Mb II using a threefold increase in protein concentration.

Autoxidation studies. Autoxidation rate measurements were made on all 3 myoglobins simultaneously. The procedure used was essentially the same as that employed by Snyder and Ayres (1961). The samples, in 0.6*M* phosphate buffer of pH 5.91, were placed in test tubes, which were submerged in water at the desired experimental temperature. Solid sodium dithionite was added to each solution to make the final concentration 0.04%. The solutions were shaken in air, transferred to cuvettes, and read spectrophotometrically at 580 mμ. The solutions were then

poured back into the test tubes, shaken, transferred back to the cuvettes, and re-read at 580 mμ. This procedure was repeated until maximum absorbancy values at 580 mμ were obtained. The cuvettes were then left in the cell compartment for the duration of the experiment. An automatic cuvette-positioning device (Wood and Gilford, 1961) moved the cuvettes every 15–17 sec. Readings at 580 mμ were taken at irregular intervals for 2–5½ hr, depending on the temperature. The temperature was controlled for different experiments at 14.5, 19.0, and 29.5°C to ±0.5°C by forcing water at the desired temperature through thermospacers placed on each side of the cell compartment. After completing a run, a few crystals of potassium ferricyanide were dissolved in each cuvette and a final reading was taken at 580 mμ. The results were converted to percentages of oxymyoglobin and plotted as described by Snyder and Ayres (1961).

RESULTS AND DISCUSSION

Absorption spectra. Fig. 1 gives the light absorption spectra for the met-derivatives of Mb I_A, Mb I_B, and Mb II, and Fig. 2 compares the spectra of the carboxy-derivatives. Both figures show that the 3 myoglobins absorb at the same wavelengths, but they differ in absorptivity values at most wavelengths. Rumen (1959) noted similar results with the multiple myoglobins of seal muscle,

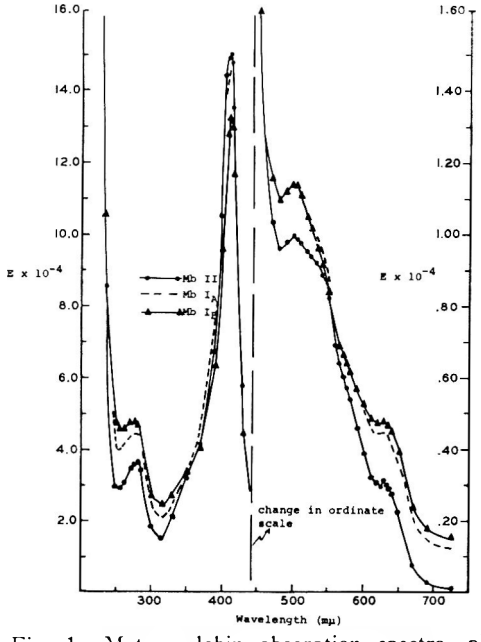

Fig. 1. Metmyoglobin absorption spectra, pH 6.90.

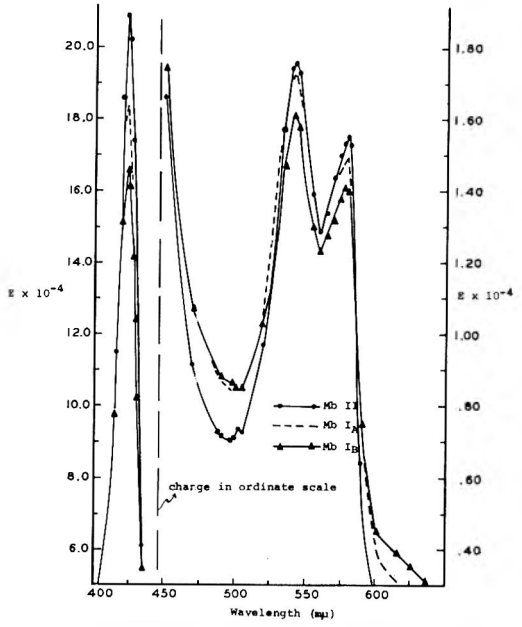

Fig. 2. Carboxy myoglobin absorption spectra.

whereas Rossi-Fanelli and Antonini (1958, 1960) and Akeson and Theorell (1960), respectively, reported that the multiple myoglobins of human, tuna, and horse muscle exhibit the same absorption spectra.

The absorption maxima of the met-derivatives of Mb I_A, Mb I_B, and Mb II occur at the wavelengths 628, 502, 410, and 278 mμ. These wavelengths agree closely with those reported for metmyoglobin preparations from the horse (Bowen, 1949), whale (Smith and Gibson, 1959), and seal (Rumen, 1959). The absorption minima of the 3 metmyoglobins, however, occur at wavelengths that suggest the presence of alkaline metmyoglobin. Since Bowen's (1949) spectrum was taken in pH 7.0 buffer and the spectrum in this study was determined in pH 6.90 buffer, the pK of the reaction acid met Mb ⇌ alkaline met Mb must be lower for beef myoglobin than that reported by George and Hanania (1952) for horse myoglobin. The spectra of metmyoglobins Mb I_A, Mb I_B, and Mb II, when taken in pH 6.50 buffer, exhibited the same wavelengths of minima absorption (590 mμ, 465 mμ) as reported for other species (Bowen, 1949; Rumen, 1959).

Absorption maxima and minima of the carboxy-derivatives of Mb I_A, Mb I_B, and

Mb II occur at the same wavelengths as reported for other species (Bowen, 1949; Smith and Gibson, 1959; Rumen, 1959). Table 1 lists the molar absorptivities of the 3 myoglobins at positions of maxima and minima and compares them with literature values.

Absorbancy ratio values may be prepared by dividing the absorbancy at one wavelength by the absorbancy at a second wavelength. In this way, calculations of molecular weight and protein content are eliminated and comparisons with values appearing in the literature become more meaningful. The absorbancy ratio values, at various wavelengths in the range of 500–600 mμ, of Mb II and Rumen's (1959) myoglobin I are very similar. Comparison of the other myoglobins in Table 1 indicates no other close similarity.

Heme content. The relative heme contents of the 3 bovine myoglobins indicated that all had the same molecular weight. The absorbancy readings at 557 mμ of the pyridine hemochromogens divided by the protein contents produced the values 1.48, 1.48, and 1.50 for Mb I_A, Mb I_B, and Mb II, respectively. The absorptivity of pyridine hemochromogen was not determined in this study, and since the literature (Lemberg and Legge, 1949) lists several, widely-varying values, the molecular weight of bovine myoglobin was not calculated.

Heme cleavage. Figs. 3 and 4 present the results of heme cleavage experimentation. Fig. 3 demonstrates the susceptibilities of the 3 myoglobins to acid cleavage. Fig. 4 presents the same data but is a logarithmic expression where A represents the absorbancy at the indicated pH.

Figs. 3 and 4 reveal that there are 2 plots presented for Mb II. The plots of Mb I_A, Mb I_B, and Mb II were made using approximately the same protein concentration. The second curve of Mb II, labeled Mb II*, was made using this protein at a threefold higher concentration. It is evident that the plots of Mb II and Mb II* are distinctly different. Since the same myoglobin sample was used in plotting both MbII and Mb II* curves and differed only in concentration, the validity of the method was questioned. The results should have been independent of concentration since they were based on percentages.

Lewis (1954) and Lewis and Schweigert (1955) determined the percent of heme cleavage at various pH values of crystalline beef myoglobin. In the second paper, the

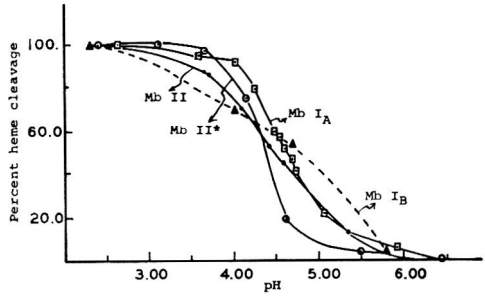

Fig. 3. Acid cleavage of heme from myoglobins.

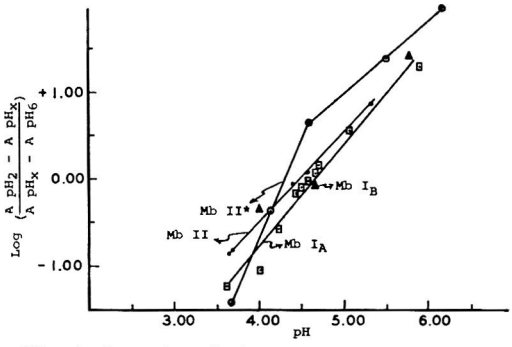

Fig. 4. Log plot of cleavage.

Table 1. Millimolar absorptivity values of the CO-derivatives of various myoglobins.

Wavelength (mμ)	Horse[a]	Whale[b]	Seal[c]	Mb I_A	Mb I_B	Mb II
578	12.9	15.1	12.8	15.1	14.1	15.5
560	10.6	.....	10.5	13.1	12.3	12.8
542	14.8	17.0	14.5	17.3	16.1	17.6
502	5.3	.....	5.0	8.5	8.5	7.3
423	.....	187	206	184	166	209

[a] Bowen (1949).
[b] Smth and Gibson (1959).
[c] Rumen (1959)—myoglobin fraction I.

same authors (1955) reported results distinctly different from those reported in the first paper (Lewis, 1954) but gave no explanation. The heme cleavage plots of Mb II* in Figs. 3 and 4 are very similar to those presented by Lewis (1954).

It was noted in this study that at high absorbancy readings, i.e. values greater than 1.00, the absorbancy increased rapidly during its determination. The absorbancy of the same sample did not increase significantly if kept in the dark. Hemin, in acetone, thus appeared to undergo a reaction dependent on concentration and light energy. The nature of the reaction was not investigated in this study and could not be found in the literature.

Autoxidation. The autoxidation rate constants (k) for the 3 myoglobins were almost identical. The rate constants for Mb I_A, Mb I_B, and Mb II at 29.5°C were respectively 0.47, 0.47, and 0.46 per hour. At 19°C the k values for all 3 myoglobins were 0.05 per hour. Although the rate constants at 19°C agreed with published values (Snyder and Ayres, 1961; Brown and Dolev, 1963), the k values at the higher temperature were all substantially greater.

Autoxidation rates were determined at various times throughout this study. It was noted that as the homogeneity of the samples increased, the autoxidation rates at higher temperatures (29–33°C) also increased. Brown and Dolev (1963) reported that crude myoglobin preparations autoxidized at a substantially slower rate than myoglobin purified by DEAE cellulose chromatography. They attributed this observation to a protective effect exerted by contaminating, and possibly sulfhydryl-containing, proteins in the preparation. The postulation of Brown and Dolev (1963), however, would not be applicable to the findings of this study. All the myoglobin fractions studied were considered to be free of other proteins, but only to vary in degree of freedom from the other myoglobins.

Also, at the higher temperatures (29–30°C) employed in this study it was noted that the greater the homogeneity of the samples, the more pronounced were anomalies in the initial rates of autoxidation. The amount of dithionite (0.04%) used in these experiments was less than the level reported by Snyder and Ayres (1961) as having an effect on the rate constant or initial readings. Fig. 5 illustrates typical autoxidation rate plots of Mb I_A, Mb I_B, and Mb II at a high temperature.

Although no measurements on the rate of oxygenation of the myoglobin was attempted, it appeared that Mb I_B was oxygenated much more rapidly than the other 2 fractions.

The possibility that the 3 myoglobins are artifacts of the isolation procedures was considered in an earlier paper (Quinn *et al.*, 1964). To the arguments previously pre-

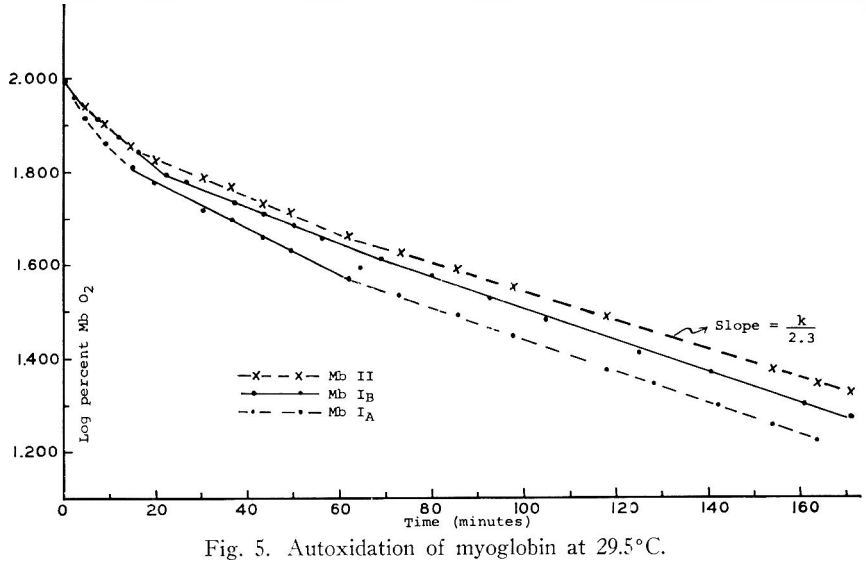

Fig. 5. Autoxidation of myoglobin at 29.5°C.

sented against the possibility of denaturation may be added the similarity of autoxidation rates. The similarity suggests that one or two of the myoglobins were not denatured, as Watts (1954) reported a relationship between denaturation and rate of autoxidation.

The myoglobins Mb I_A, Mb I_B, and Mb II differed in spectral absorptivity values and ease of heme-globin cleavage. If the heme cleavage results for the 3 myoglobins at similar concentrations are valid, then the heme-globin linkages of the myoglobins would appear to be different. The spectral results confirm this difference. The replacement of a single amino acid in the main portion of the globin by another amino acid, as in the difference between normal and sickle-cell hemoglobin (Ingram, 1957), or the slight rearrangement of a segment of the polypeptide chain, as was found for horse myoglobin (Akeson and Theorell, 1960), would not alone account for the spectral differences observed. The similarity of the wavelength positions of minima and maxima absorption among the 3 myoglobins indicates a similarity of the main heme linkages, i.e., the iron-globin bonds (Brill and Williams, 1961). The differences in absorptivity values imply dissimilarities in the weaker heme linkages, i.e., the porphyrin-globin bonds. Brill and Williams (1961) found a decrease in Soret range absorptivity to result from an attack on the porphyrin ring of a heme protein.

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A Method for Determining 5'-Nucleotides [a]

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(Manuscript received June 17, 1963)

SUMMARY

A method is described for the separation and quantitative determination in a variety of foods of the following 5'-nucleotides; cytidine-5'-phosphate, adenosine-5'-phosphate, uridine-5'-phosphate, inosine-5'-phosphate, and guanosine-5'-phosphate. This procedure employs a Dowex 1 ion-exchange resin in the formate form to adsorb and concentrate the nucleotides from an aqueous extract of the food sample. The nucleotides are separated and eluted in the order previously given by means of a gradient elution system, consisting of water–formic acid–sodium formate. The method gives complete resolution of the 5'-nucleotides from each other, but not from their corresponding 2'- and 3'-forms. Therefore, following chromatographic separation, the 5'-nucleotides are determined colorimetrically in the presence of the 2'- and 3'-nucleotides by oxidation with periodate and reaction of the oxidation products with 2,4-dinitrophenylhydrazine.

The flavor-enhancing properties of 5'-nucleotides have become increasingly apparent in recent years (Kuninaka, 1960), and studies on the occurrence of these compounds have required the development of sensitive and accurate methods of analysis. Many of the methods for determination of the 5'nucleotides (Bergkvist and Deutch, 1954; Fujita *et al.*, 1959; Jones and Murray, 1960; Nakajima *et al.*, 1961a; Nakajima *et al.*, 1961b; Okuhara and Aso, 1959; Saito and Arai, 1958; Yamada *et al.*, 1962) are based on ion-exchange chromatographic separation and spectrophotometric determination of these compounds from their ultraviolet absorption spectra. Unfortunately, these methods are not specific in that the 2'(3')-nucleotides interfere in the analysis. The reason is that these compounds are eluted by ion-exchange chromatography at or near the position of their corresponding 5'-isomers and exhibit identical ultraviolet-absorption spectra (Nakajima, 1961a). Since the 2'(3')-nucleotides can arise by chemical or enzymatic degradation of ribonucleic acid, their effects can be considerable in analysis of the minute amounts of 5'-nucleotides that occur in most food products. Consequently, corroborative identification is required to establish their presence or absence in the eluted fractions. This takes time and makes these techniques somewhat unsuited for routine purposes.

To assess the flavorful effects of 5'-nucleotides, a specific and sensitive method for determination of these compounds was needed that could be applied routinely to food products. This paper describes a method for the separation and quantitative determination of cytidine-5'-monophosphate (5'CMP), adenosine-5'-monophosphate (5'AMP), uridine-5'-monophosphate (5'UMP), inosine-5'-monophosphate (5'IMP), and guanosine-5'-monophosphate (5'GMP). The procedure employs a Dowex 1 ion-exchange resin in the formate form to adsorb and concentrate the nucleotide from an aqueous extract of the food sample. The nucleotides are separated and eluted in an elution system consisting of water-formic acid-sodium formate. The method gives complete resolution of the 5'-nucleotides from each other but not from their corresponding 2'(3')-forms. Therefore, following chromatographic separation, the 5'-nucleotides are determined colorimetrically in the presence of the 2'- and 3'-nucleotides by oxidation with periodate and reaction of the oxidation products with 2,4-dinitrophenylhydrazine (2,4-DNPH).

[a] Presented at the 23rd Annual Meeting of the Institute of Food Technologists, Detroit, Michigan, May 28–29, 1963.

EXPERIMENTAL

The 5'-nucleotides used were obtained from various commercial sources. Their purity was determined by comparison of molar absorptivity values with those reported in the literature (Bock *et al.*, 1956), and also by quantitative oxidation with periodic acid according to the procedure of Dyer (1956). The purity of the 5'-nucleotides by these methods averaged 99.3%.

All other reagents and chemicals were C.P. or the best commercial grade available.

Chromatographic solutions. 0.5*N* formic acid and 0.2*N* sodium formate.

Colorimetric reagents. *Periodic acid.* A stock solution of 0.1*N* periodic acid was prepared by dissolving 2.85 g of paraperiodic acid (H_5IO_6) in water and diluting to 250 ml. This solution is sensitive to light and heat, but at room temperature in the dark is stable for several weeks. A 1:10 dilution of the stock solution was used for analysis.

Sodium arsenite. A stock solution of 0.05*N* sodium arsenite (Na_2HAsO_3) was prepared by dissolving 2.50 g of arsenious oxide in 50 ml of 20% sodium hydroxide and adjusting the volume to 1 L.

2,4-DNPH. A saturated solution of 2,4-DNPH was prepared by adding 20 g of the reagent to 200 ml of 2*N* hydrochloric acid. The solution was shaken vigorously, allowed to stand overnight, and then filtered. The reagent is stable indefinitely but should be filtered before each use.

Instrumentation. A Vanguard Automatic UV analyzer was used to monitor the ultraviolet absorbing components eluted from the chromatographic column. All UV spectrophotometric data were obtained with a Cary Model 14 spectrophotometer. Colorimetric determinations were made with the Beckman Model B spectrophotometer.

METHODS

Extraction of nucleotides. The sample to be analyzed for 5'-nucleotide content was blended until homogeneous (1–2 min) in a Waring blender. Five to 10 g of sample were weighed into a 250-ml Erlenmeyer flask, and the volume was adjusted with boiling water to approximately 50 ml. The flask was stoppered and the contents shaken vigorously for several minutes in order to extract the nucleotides. Quantitative transfer of the sample was made to centrifuge tubes, and the aqueous phase was collected by centrifugation. The sediment was resuspended in a minimum amount of water and centrifuged, and the clarified extracts were combined. The total volume did not exceed 75 ml. To precipitate proteinaceous material, 10 ml of a 20% solution of trichloroacetic acid (TCA) and 30 ml of 95% ethanol were added to the aqueous extract. The mixture was clarified by centrifugation, and the supernatant was transferred to a separatory funnel. The TCA and alcohol were removed by three successive extractions with equal volumes of diethyl ether. The aqueous phase was separated, adjusted to pH 8.0 with 5*N* sodium hydroxide, and diluted to exactly 100 ml.

Chromatographic separation of nucleotides. Dowex 1 anion-exchange resin (200–400-mesh) was washed with water to remove suspended solids and packed as a wet slurry in a 12-mm ID glass column to a height of approximately 160 mm. The column was fitted with a stopcock and a plug of glass wool to retain the resin. The resin was placed in the hydroxyl form by treatment with three bed volumes of 1*N* sodium hydroxide. After the resin was washed free of alkali with distilled water, it was placed in the formate form by addition of three bed volumes of 6*N* formic acid. The excess reagent was removed by washing with water. An aliquot of test solution (5–25 ml) was added to the top of the column, and passed through at the rate of 1 ml per min. The resin was washed free of the UV-absorbing materials with water until the effluent read approximately 100% transmission at 260 mμ.

Elution. The nucleotides were eluted by a gradient of water, formic acid (0.5*N*), and sodium formate (0.2*N*). A 500-ml aspirator bottle containing 250 ml of water was attached to the upper portion of the chromatographic column. A second aspirator bottle containing 250 ml of 0.5*N* formic acid was connected above the first container. To this second bottle was attached a 500-ml separatory funnel containing 500 ml of 0.2*N* sodium formate. Connections between these vessels were closed with stopcocks, and the solutions in the bottles were mixed with magnetic stirrers. Fig. 1 shows a photograph of the assembly.

To begin the elution, the stopcocks were opened, the solutions mixed and allowed to percolate through the column at a flow rate of 1 ml per minute. The effluent from the column then passed through a UV analyzer, where the absorbance at 260 mμ was recorded automatically. This enabled the fractions containing the 5'-nucleotides to be located easily in the 5-ml portions that were collected. These fractions were then composited, diluted to constant volume (100–200 ml), and examined by UV spectrophotometry to characterize the nucleotide present. A portion of this solution was then analyzed by the periodate–2,4-dinitrophenylhydrazine procedure to determine the concentration of the 5'-nucleotide actually present.

HIO_4–2,4-DNPH colorimetric analysis. To a 5-ml portion of the test solution was added 1 ml of 0.01*N* HIO_4 and the solution heated for exactly 3 min at 70°C. This period was found to be very critical, since longer reaction times (5 min or more) resulted in over-oxidation of the nucleotide. The

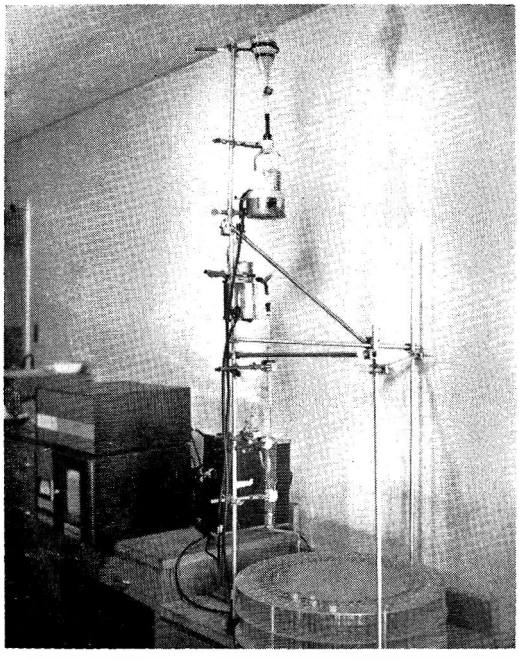


Fig. 1. Apparatus for chromatograph separation of 5′-nucleot.des.

oxidation was stopped by the addition of 1 ml of 0.05*N* sodium arsenite (Na_2HAsO_3). This was followed immediately by the addition of 1 ml of concentrated hydrochloric acid and 1 ml of the 2,4-dinitrophenylhydrazine (2,4-DNPH) reagent. After mixing, the tubes were placed in a boiling-water bath for 10 minutes to hydrolyze the oxidation products and to facilitate the precipitation of the bis-hydrazone. It was found necessary to add the reagents in the order described to prevent interaction of periodic acid and 2,4-DNPH, which tended to give very high reagent blanks. The precipitate was collected on a medium-porosity fritted-glass filter and washed several times with water to remove traces of unreacted 2,4-DNPH reagent. The bis-2,4-DNPH derivative was then dissolved in acetone and diluted to volume, and the absorbance of the yellow solution was read at 435 mμ in a 10-mm Pyrex absorbance cell against a reagent blank. Since the extinction coefficient of each 5′-nucleotide as determined by the colorimetric procedure was essentially the same (Table 1), the nucleotide content of each fraction was calculated from a single standard. This standard was equivalent to 2 μg/ml 5′IMP in the final acetone solution.

RESULTS

Fig. 2 shows the chromatographic separation of a synthetic mixture of five 5′-nucleotides (5′CMP, 5′AMP, 5′UMP, 5′IMP, and 5′GMP) and the order in which these compounds are eluted by the gradient elution system.

Table 1. Colorimetric molar absorptivities of the 5′-nucleotide by periodate-2,4-DNPH reaction.

5′-nucleotide	$a_m \times 10^{-3}$ [a]	SD [b]
5′CMP	93.8	±0.7
5′AMP	92.1	±0.8
5′UMP	94.5	±0.2
5′IMP	91.5	±1.0
5′GMP	88.6	±0.9

[a] Molar absorptivity.
[b] Values given are the average of ten determinations ±std. dev.

Fig. 3 shows the elution pattern of the 5′-nucleotides when chromatographed together with a mixture of 2′(3′)-nucleotides obtained from the alkaline hydrolysis of ribonucleic acid (Hutchinson and Munro, 1961). It is evident that the first nucleotides to be eluted, CMP and AMP, are still well separated, although there is some overlap of the 2′(3′)-compounds with the 5′-isomer. In the case of the more retentive nucleotides, UMP, IMP, and GMP, the various isomers are completely unresolved. This is in conformity with the works of Volkin and Cohn (1954) and Nakajima *et al.*

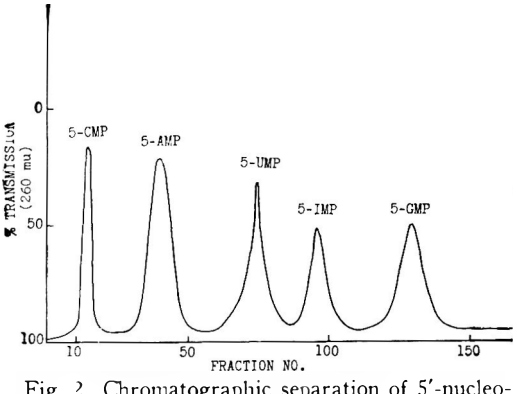


Fig. 2. Chromatographic separation of 5′-nucleotides (Dowex 1: water–0.5*N* formic acid–0.2*N* sodium formate).

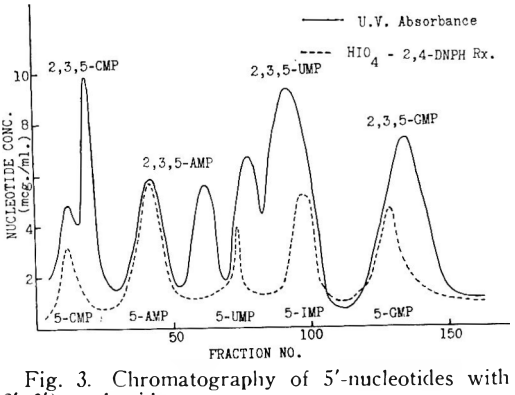


Fig. 3. Chromatography of 5′-nucleotides with 2′(3′)-nucleotides.

(1961a) regarding the elution positions of the 2′-, 3′-, and 5′-nucleotides in formic acid–sodium formate systems, and illustrates the interference possible from the 2′(3′)-isomers in this type of analytical scheme.

The 5′-nucleotides are distinguished from the 2′- and 3′-nucleotides by being selectively oxidized with periodic acid (Baddiely, 1955). Table 2 demonstrates the manner in which this reaction can be used together with 2,4-dinitrophenylhydrazine to determine the 5′-isomer in a mixture with the 2′(3′)-compounds. Fig. 3 shows that this reaction can also be employed to locate the positions at which the 5′-nucleotides are eluted from the column.

Table 2. Colorimetric determination of 5′AMP in presence of 2′AMP and 3′AMP (periodate–2,4-DNPH R_x).

Nucleotide	Conc. (μg/ml)	Absorb. (435 mμ)
5′AMP	2	0.49
3′AMP	2	<0.01
2′AMP	2	<0.01
5′AMP + 2′AMP + 3′AMP	6	0.50

Tables 3 and 4 show recovery by the HIO_4-2,4-DNPH method of individual 5′-nucleotide chromatographed with a mixture of the 2′- and 3′-nucleotides. These data were obtained by combining those fractions containing individual nucleotides, characterizing these by ultraviolet-absorption spectroscopy, and analyzing an aliquot of this composite colorimetrically for 5′-nucleotide as previously described.

In some cases the nucleotide content of the fractions was too dilute to permit their characterization by UV analysis. However, repeated runs on known mixtures of nucleotides established the positions at which each of the 5′-nucleotides was eluted. Consequently, the individual nucleotides could be located and determined by reacting each fraction with periodic acid and 2,4-dinitrophenylhydrazine. The development of the osazone precipitate in these fractions thus indicated the position of the individual 5′-nucleotide, and the precipitates could then be composited, dissolved in acetone, and analyzed colorimetrically. In this manner concentration of 5′-nucleotides as low as 25 ppm could be determined by this procedure.

APPLICATION TO FOODS

To establish the applicability of the present method to the determination of 5′-nucleotides in foods, several products represented as having significant nucleotide levels were selected for analysis. Table 5 depicts the results of application of this

Table 3. Recovery of 5′-nucleotides by chromatographic separation and colorimetric analyses.

	Tube no.	Amount added (mg)	Recovered		Amount added (mg)	Recovered		Amount added (mg)	Recovered		Amount added (mg)	Recovered	
			mg	%		mg	%		mg	%		mg	%
5′CMP	11–17	0.20	0.19	95	0.50	0.46	92	1.00	0.94	94	2.00	1.97	99
5′AMP	34–50	0.20	0.21	105	0.50	0.51	103	1.00	1.04	104	2.00	2.10	105
5′UMP	68–81	0.20	0.19	93	0.50	0.46	93	1.00	0.99	99	2.00	1.90	95
5′IMP	88–107	0.20	0.20	98	0.50	0.52	105	1.00	1.02	102	2.00	1.97	99
5′GMP	114–144	0.20	0.19	95	0.50	0.49	97	1.00	0.96	96	2.00	1.90	95

Table 4. Recovery of 5′-nucleotides from chromatographed mixtures containing 2′(3′)-nucleotides (periodate–2,4-DNPH colorimetric analysis).

5′-Nucleotides	Tube no.	Added (mg)	Recovered	
			mg	%
CMP	11-17	1.98	1.90	96
AMP	34-50	1.83	1.94	106
UMP	68-81	2.03	1.82	90
IMP	88-107	1.88	1.84	102
GMP	114-144	2.18	2.18	100

method to determination of the 5′-nucleotide content of these products. Also shown are the fractions in which each component nucleotide was eluted, the UV-absorption maximum, and reactivity of these fractions toward periodic acid–2,4-dinitrophenylhydrazine. Also given, for comparison, is the concentration of 5′-nucleotide as determined by UV analysis.

As shown in Table 5, all of the products analyzed contain 5′IMP as the major 5′-nucleotide. Except for fresh tuna, these products also contain 5′AMP as a minor constituent. Only fresh beef was shown to contain 5′CMP; the concentration was higher (0.009%) by UV analysis than by colorimetric analysis (0.003%). This is indicative of the greater specificity of periodic-2,4-DNPH method and suggests the presence in this fraction of other UV-absorbing substances that do not react with these reagents. This can be illustrated more particularly in the case of fresh tuna, in which a component eluted in the position of 5′CMP, and having an absorption maximum corresponding to this compound, did not react in the colorimetric procedure. A second component similar in UV characteristics and elution pattern to 5′AMP was also unreactive. Both components, which were tentatively identified as 2′(3′)-CMP and 2′(3′)-AMP, are not distinguished from the 5′-compound by UV analysis, and so were determined as the 5′-compound by this method.

Table 6 shows the results obtained when known amounts of 5′IMP were added to these products

Table 5. 5′-Nucleotide content of foods (UV vs. periodate–2,4-DNPH analysis).

	Frac. no. (5 ml)	Max. (mμ)	HIO_4–2,4-DNPH (R_x)	Ident.	Concentration (%)[a]	
					UV[b]	Color[c]
Fresh beef	9–14	268	Pos.	5′CMP	0.009	0.003
	25–35	258	Pos.	5′AMP	0.004	0.003
	68–80	262	Pos.	5′UMP	Trace	Trace
	87–113	249	Pos.	5′IMP	0.086	0.083
		Total 5′-nucleotide content			0.099	0.089
Fresh chicken	31–39	260	Pos.	5′AMP	Trace	0.002
	82–90	262	Pos.	5′UMP	Trace	0.003
	101–131	249	Pos.	5′IMP	0.233	0.234
		Total 5′-nucleotide content			0.233	0.239
Fresh tuna	9–14	270	Neg.	2′(3′)CMP	0.002	0.000
	39–55	260	Neg.	2′(3′)AMP	0.001	0.000
	101–138	249	Pos.	5′IMP	0.200	0.180
		Total 5′-nucleotide content			0.203	0.180
Cooked beef	18–31	258	Pos.	5′AMP	0.026	0.019
	62–82	249	Pos.	5′IMP	0.046	0.049
		Total 5′-nucleotide content			0.072	0.068
Cooked chicken	13–20	260	Pos.	5′AMP	0.010	0.009
	53–70	250	Pos.	5′IMP	0.057	0.054
		Total 5′-nucleotide content			0.067	0.063
Cooked tuna	8–15	249	Pos.	Unknown	.....	.....
	25–37	258	Pos.	5′AMP	0.005	0.005
	84–120	249	Pos.	5′IMP	0.200	0.180
		Total 5′-nucleotide content			0.205	0.185

[a] As anhydrous disodium salt.
[b] UV spectrophotometric analysis.
[c] Colorimetric analysis, periodate–2,4-DNPH.

Table 6. Recovery of 5'IMP from food products.

	5' IMP				
	Present (mg)	Added (mg)	Found (mg)	Recovery mg	Recovery %
Fresh tuna	0.88	0.75	1.65	0.77	103
Cooked tuna	0.91	0.75	1.64	0.73	97
Fresh beef	2.08	0.75	2.81	0.73	97
Cooked beef	1.23	0.75	1.98	0.75	100
Fresh chicken	1.17	0.75	1.91	0.74	99
Cooked chicken (freeze-dried)	0.78	0.75	1.48	0.70	93

and recovery of this compound determined by chromatographic separation and colorimetric analysis.

INTERFERENCES

Any compound that reacts with periodic acid and/or 2,4-DNPH could conceivably interfere in colorimetric determination of the 5'-nucleotides. However, since this method incorporates the use of anion-exchange chromatography as a prerequisite to colorimetric analysis, only anionic compounds are retained by the resin. As a result, the interference from carbonyl compounds, carbohydrates, nucleosides, or other nonionic substances is eliminated since these compounds are washed from the resin prior to gradient elution.

Other compounds of a biological origin with anionic groupings, such as phosphorylated sugars and the 5'-di- and triphosphonucleotides, because of the presence of the vicinal hydroxyl group, interfere in the method. In the latter case, however, the di- and triphosphonucleotides are not eluted by the gradient employed. The phosphorylated sugars, as shown in Fig. 4, are eluted in the vicinity of the 5'-nucleotides although for the most part still in separate fractions. However, these compounds do not absorb in the ultraviolet, and in comparison with 5'-nucleotides are not as reactive in the colorimetric procedure (Table 7). It would seem, therefore, that periodate oxidation in combination with ion-exchange chromatography and ultraviolet spectroscopy, restricts the method to the 5'-nucleotides and in this manner is highly specific for the determination of these compounds.

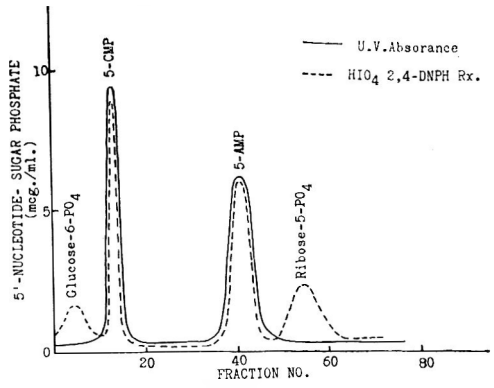


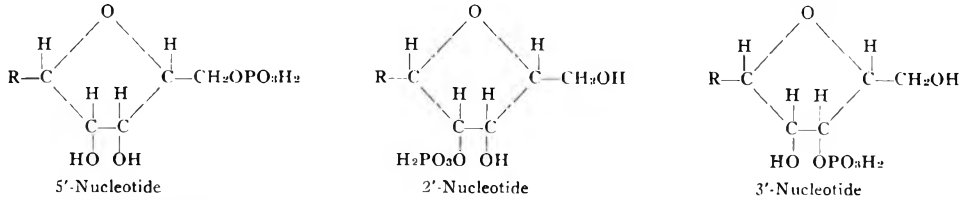
Fig. 4. Chromatography of 5'-nucleotides with sugar phosphates.

Table 7. Relative color intensities of 5'AMP and the sugar phosphates by periodate–2,4-DNPH reaction.

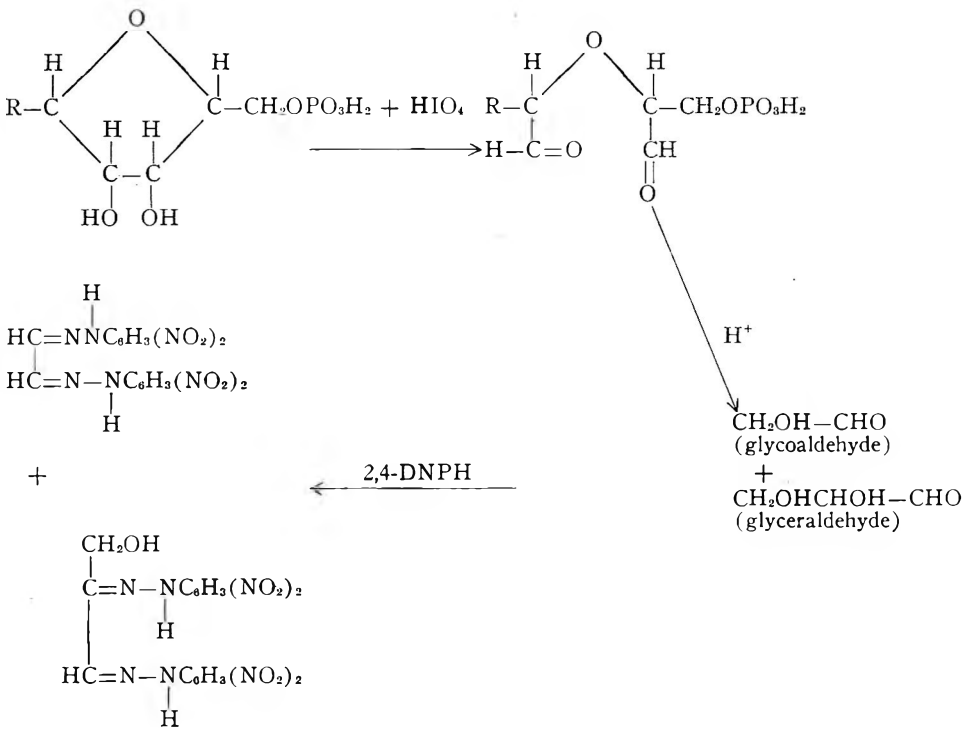
	Conc. (μg/ml)	Abs.
5'AMP	1.0	0.240
Ribose-5-phosphate	1.0	0.070
Glucose-6-phosphate	1.0	0.010

DISCUSSION

The 5'-nucleotides are distinguished from both the 2'- and 3'-nucleotides by the vicinal hydroxyl groups between the 3rd and 4th carbon atoms of the ribose moiety.



The selective oxidation of adjacent hydroxyl groups by periodic acid is well recognized, and the latter reaction has been utilized by Davoll *et al.* (1946) and Cohn (1960) and Khym (1960) in elucidating the structure of the 5'-nucleotides and 5'nucleosides. In the present study, this reaction has been used to provide a specific method for determining the latter type of compounds in the presence of the 2'- and 3'- isomers. In this scheme, the products of periodate oxidation are reacted with 2,4-DNPH and estimated colorimetrically according to the following reaction sequence:



Khym and Cohn (1960) showed that the products of periodate oxidation of the nucleosides are glycolaldehyde and glyceraldehyde. It is also probable that these same compounds are formed as a result of periodate oxidation of the 5′-nucleotides. This would account for the ability of the present method to determine concentrations of nucleotides lower than could be detected by direct UV spectrophotometric analysis since, as shown above, the addition of 4 moles of 2,4-DNPH/mole of 5′-nucleotide increases the total mass of the derivative and therefore makes the reaction very sensitive. Even greater sensitivity is achieved in the analyses by developing the blue color of the osazone with alkali and measuring this colorimetrically (Table 8). For practical purposes, however, the colorimetric determination of the osazone at 435 mμ should suffice for most routine analyses.

Table 8. Comparison of molar absorptivities of 5′-nucleotides by UV spectrophotometric and HIO_4–2,4-DNPH colorimetric methods.

	$a_m \times 10^{-3}$		
	UV (pH 7)	Colorimetric	
		Yellow [a]	Blue [b]
5′CMP	9.1	93.8	121.9
5′AMP	15.4	92.1	129.7
5′UMP	10.0	94.5	122.9
5′IMP	12.2	91.5	119.0
5′GMP	13.7	88.6	115.2

[a] 2,4-DNPH derivative in acetone.
[b] 2,4-DNPH derivative in 9:1 acetone–2% KOH/methanol.

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Changes in Concentrated Milk During Frozen Storage[a]

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(Manuscript received October 14, 1963)

SUMMARY

Prefreezing treatments greatly influence the physical stability of concentrated milk during frozen storage. Treatments that tend to retard lactose crystallization extend storage life. Agitation or cooling procedures that induce nucleation accelerate destabilization, as does the presence of foreign nuclei. Effluent diagrams from a DEAE column show changes in the casein fractions and in β-lactoglobulin. The mechanism of protein insolubilization during frozen storage appears to be more complex than previously suggested.

The preservation of milk by frozen storage has been of industrial and academic interest for many years, and the volume of literature on the subject is consequently considerable. Samuelson *et al.* (1957) summarized the state of the problem at the time of their review. One of the principal defects encountered in the product is a deterioration in physical stability. Early thawing and reconstitution yields a product indistinguishable from normal fluid milk, but during longer frozen storage the proteins become unstable, and when the milk is thawed and diluted to normal concentration the proteins settle out. This milk, besides being unsightly, has an undesirable, chalky texture, making it an unsuitable beverage.

In recent years it has become increasingly apparent that lactose crystallization is intimately associated with the instability of frozen concentrated milk (Tumerman *et al.*, 1954; Rose and Tessier, 1956; Tessier *et al.*, 1956; Desai *et al.*, 1961; Winder, 1962; Johnson and Tumerman, 1962; Wells and Leeder, 1963). As a general rule, as long as the lactose remains in solution the proteins remain stable, redispersing as in normal milk when thawed and reconstituted. When the lactose crystallizes, however, instability develops rapidly. Recent work of Johnson and Tumerman (1962) indicates, however, that this relation between lactose crystallization and protein instability may not hold for milks having less than 30% solids. Thus it is evident that the role of lactose is still unexplained.

The present work was carried out to evaluate some of the factors influencing the stability of proteins in frozen milks and to measure some of the protein changes.

EXPERIMENTAL PROCEDURE

Both concentrated whole milk and skim milk were used. They were prepared by pasteurization and condensing by commercial procedures to 32% total solids for the nonfat product and 36.5% total solids (10% fat) for the whole milk. The milk was preheated to 165°F and held for approximately 45 sec before entering a Mojonnier double-effect pan operating at 158 and 105°F, respectively. In experiments concerned with lactose crystallization, the condensed milk was heated 25 min at 155°F to destroy the crystal nuclei, cooled to 100°F, and packaged in polyethylene tubing of 1 in. diameter. The tubing was closed at both ends with Cryovac clamps. The milk was frozen at −10°F for at least 24 hr before being stored at 10–15°F.

At various storage intervals a portion of the frozen milk was removed, diluted to the normal concentration of fluid milk, and centrifuged under standard conditions to determine the solubility of the milk proteins (Desai *et al.*, 1961). The volume of precipitate was measured in graduated centrifuge tubes and expressed as milliliters of precipitate per 100 ml of reconstituted milk.

To measure changes in the soluble proteins, the supernatant was analyzed by the procedure of Yaguchi *et al.* (1961). The supernatant was dialyzed in the cold against large volumes of 0.02*M* sodium phosphate at pH 7.0 for two days before chromatographic analysis. The proteins were separated using the stepwise elution procedure from the DEAE column. The milk used in this experiment was heated 10 min at 100°F, packaged, and

[a] Supported in part by a USPHS grant (EF 00094) and by the California Dairy Council. Presented at the Sixth Milk Concentrates Symposium, April 10, 1963, Davis, California.

immediately frozen at −10°F. It showed better than normal stability during storage at 10–15°F.

RESULTS AND DISCUSSION

It has been well known since work of Ostwald and Luther (1902) that agitation of a supersaturated solution may induce nucleation and subsequent crystal growth. On the other hand, quick cooling of a supersaturated solution without agitation may result in a system that remains stable for a long period without crystallization occurring. The data in Table 1 illustrate application of this principle in the preparation of frozen concentrated milk.

Cooling with agitation greatly accelerated lactose nucleation, crystallization, and subsequent insolubilization of the proteins. Quiescent cooling to a low temperature prior to freezing improved storage stability further, as did slow cooling and freezing in the insulated box. This may be due to the establishment of a more favorable ionic equilibrium, as suggested by Van Kreveld and Van Minnen (1955) and Johnson and Tuerman (1962), but it may also include the establishment of a more stable lactose solution, since holding at lower temperatures favors a lower percentage in the alpha form.

The data in Table 1 also show several other significant points. It can be calculated that the aqueous phase of milk condensed 3:1 becomes saturated with respect to lactose at 80–100°F. Agitation during cooling, therefore, accelerated destabilization since agitation of a supersaturated solution initiates nucleation. Rapid freezing after agitated cooling reduced the rate of destabilization, presumably by reducing the time during which nuclei could form. Although the agitation initiates nucleation, the data indicate that additional formation and growth occurs while the milk is frozen following agitated cooling. Under quiescent freezing conditions the rate of freezing was not an important factor governing stability. This would be expected, since the stability of the supersaturated lactose solution would not be affected appreciably by the cooling rate. In other experiments, dry ice and liquid nitrogen were used to give very rapid freezing, but storage stability was not improved. This is in agreement with findings of Rose and Tessier (1954).

An important characteristic to recognize in studying frozen milk is the variability in crystal form and in extent of crystallization that may occur. For example, Fig. 1 is a photograph of three polyethylene tubes of frozen milk; one is essentially free of visible crystal clumps, one has a few very large

Table 1. Effect of quiescent and agitated cooling and different freezing rates on subsequent stability of frozen condensed skimmilk (32% TS) at 15°F.

Cooling conditions	Frozen at −10°F in:	Stability (ml ppt/100 ml milk)		
		11 days	19 days	34 days
Quiescent to 100°F	Glycol	0.5	4.0	18
Quiescent to 100°F	Air	0.5	4.0	20
Quiescent to 100°F	Insulated box	0.5	4.0	15
Quiescent to 80°F	Glycol	0.5	2.0	16
Quiescent to 80°F	Air	0.5	3.5	17
Quiescent to 80°F	Insulated box	0.5	3.0	14
Quiescent to 60°F	Glycol	0.5	1.5	11
Quiescent to 60°F	Air	0.5	2.0	12
Quiescent to 60°F	Insulated box	0.5	2.0	15
Quiescent to 40°F	Glycol	0	1.5	13
Quiescent to 40°F	Air	0.5	2.0	10
Quiescent to 40°F	Insulated box	0.5	2.0	11
Agitated to 60°F	Glycol	9	24	20
Agitated to 60°F	Air	11	25	22
Agitated to 60°F	Insulated box	20	26	23
Agitated to 40°F	Glycol	14	24	22
Agitated to 40°F	Air	18	23	23
Agitated to 40°F	Insulated box	28	22	24

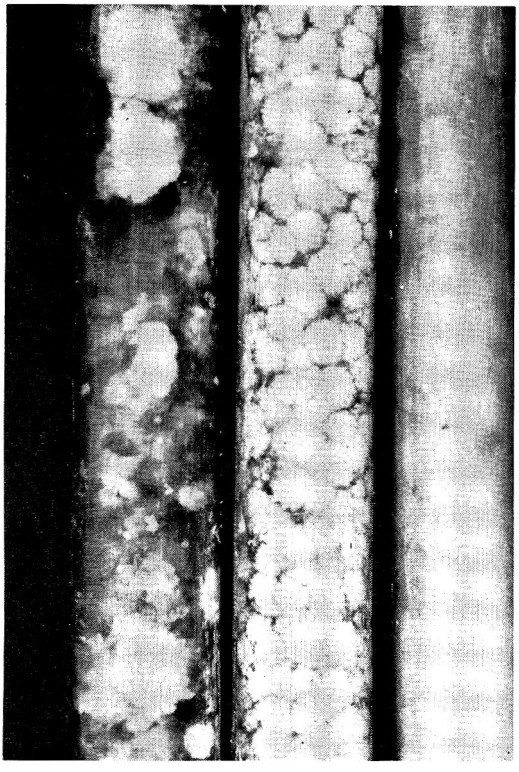

Fig. 1. Lactose crystallization in 3 samples of frozen concentrated milk.

clumps scattered here and there, and the center sample shows a great many crystal clumps have developed throughout the tube. It is easy to see how a small sample for microscopic examination could give an erroneous picture of the crystallization pattern, because the system is heterogeneous and a small sample is not an accurate composite.

One explanation for this irregular crystallization observed in frozen milk samples is that foreign nuclei present in the milk may act as crystal centers to initiate lactose crystallization. This line of reasoning has been used for many years to explain erratic crystallization of supersaturated solutions of many kinds (Ostwald and Luther, 1902) and to explain the improved stability of small samples (less chance of foreign nuclei being present). The data in Table 2 illustrate this principle. Condensed skimmilk was heated 10 min at 110°F to dissolve any crystals (calculated saturation at 80°F). One portion was not treated further and served as a control. Another portion was passed through a Sharples supercentrifuge

Table 2. Effect of nuclei on stability of frozen condensed skimmilk stored at 15°F.

	Stability (ml ppt/100 ml milk)		
	5 days	14 days	30 days
A) Condensed skim (33.33% TS)	0	0	17.0
B) Supercentrifuged condensed skim (30.5% TS)	0	0	0
C) Condensed skim and sediment from B (35.2% TC)	0.5	3.0	23.0

at 18,000 rpm at approximately 1 lb/min to remove some of the nuclei. The sediment collected in this manner was redispersed in a third portion of milk. The data show that the high-speed centrifugation improved the stability of the product and conversely, addition of the removed sediment accelerated destabilization appreciably, because the added nuclei accelerated crystallization. The sediment recovered by centrifugation was primarily protein and the accelerated lactose crystallization that resulted from its addition or retarded crystallization due to its removal can be explained best on the basis of foreign nuclei.

Since other means of inhibiting lactose crystallization have improved storage stability, i.e. hydrolysis or removal of lactose (Tumerman, *et al.*, 1954) or addition of sugars (Rose, 1956; Desai *et al.*, 1961; Wells and Leeder, 1963), it seemed reasonable that gums might also prove effective, because it had been previously shown that lactose crystallization in ice cream was inhibited or prevented by the presence of certain marine or vegetable gums (Nickerson, 1962). In the present study many purified gums as well as many commercial stabilizer formulations were investigated. In some instances, lactose crystallization was inhibited but storage stability, as measured by the solubility index, did not improve appreciably. This appears to be due to interactions of the gums and milk proteins during storage that result in precipitation. In some cases, stabilizers improved physical or flavor stability, or both, but additional studies are needed to clarify their action and effectiveness in frozen concentrated milk.

The nature of the changes occurring in the proteins of milk during frozen storage has not been studied extensively, although it is accepted that the flocculent protein precipitate that forms is primarily the caseinate system (Webb and Hall, 1935; Doan and Warren, 1947; Christianson, 1953; Wells and Leeder, 1963). Electrophoretically, no differences have been detected between normal and precipitated casein (Christianson, 1953; Wells and Leeder, 1963).

Effluent diagrams in Fig. 2 show the protein distribution in the original milk (A), determined by column chromatography (Yaguchi *et al.*, 1961), and changes in the chromatograph *(B,C,D)* of the supernatant liquid (obtained from the solubility test) as the proteins became progressively more destabilized. The initial changes were greatest in the β-lactoglobulin peaks (*f* and *g*) and one of the α-casein peaks *(m)*. As destabilization progressed, there was a general decrease in the casein region, which is in accordance with the literature. The α-, β-, and γ-caseins, however, did not decrease uniformly. Unfortunately, κ-casein is eluted only in the last effluent, when the remaining protein residue is removed from the column with NaOH. Therefore, changes in κ-casein cannot be measured by this procedure.

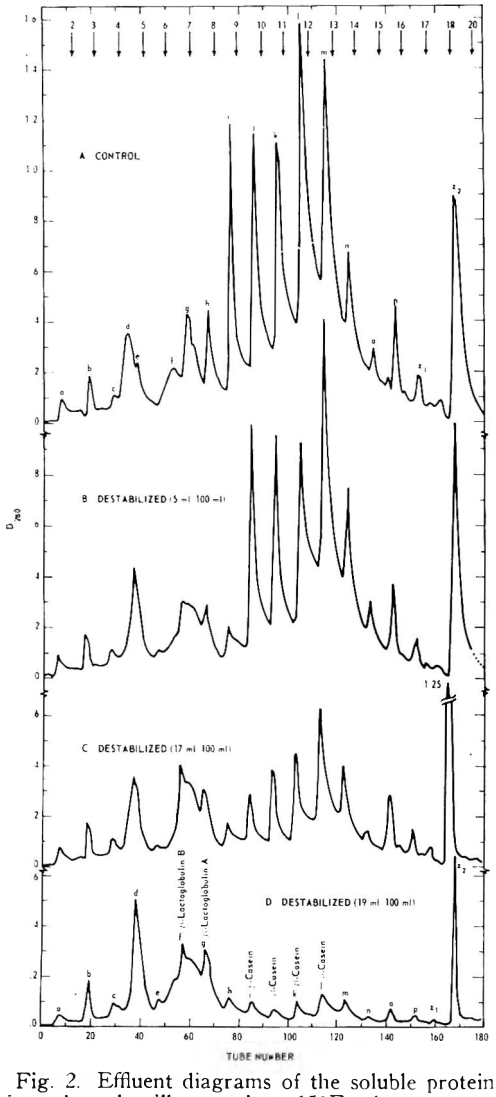

Fig. 2. Effluent diagrams of the soluble proteins of condensed milk stored at 15°F when removed from a DEAE column by stepwise elution.

The whey protein components changed little during storage, except for the β-lactoglobulins. β-Lactoglobulin A appeared to undergo more change than β-lactoglobulin B. This is of special interest since β-lactoglobulin B has been shown to be denatured by heat more readily than β-lactoglobulin A (Gough and Jenness, 1962). That changes in the caseins and whey fractions occur during frozen storage has also been shown, by starch-gel electrophoresis (Desai and Nickerson, 1964). These data indicate that the mechanism of protein insolubilization during frozen storage is more complex than previously suggested.

ACKNOWLEDGMENT

The author gratefully acknowledges the technical assistance of E. E. Moore, Ethel L. King, and H. G. Hunziker.

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Apple Juice Volatiles[a]

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SUMMARY

Thirty compounds in the volatile fraction of McIntosh apple juice were isolated and identified. Material for gas chromatography was prepared by concentrating volatiles tenfold in a rising film evaporator and extracting with ethyl chloride. The compounds were identified by comparing retention volumes and infrared spectra with those of known compounds. Volatile components tentatively identified on the basis of their infrared spectra and retention data included 4 aldehydes, 1 ketone, 11 alcohols, 10 esters, and 4 fatty acids. In addition, 7 peaks were found but not identified. A variety of column packings and operating conditions were necessary to obtain reasonable separation of the components isolated.

The data herein, on the volatile components of apple juice, augment data published elsewhere on volatiles of apple wine (Matthews *et al.*, 1962; Sugisawa *et al.*, 1962). These studies, together with those not yet completed on volatile degradation and build-up during fermentation, should provide a basis for the definition and differentiation of desirable and undesirable flavors in apple wine.

As early as 1920, odorous constituents of several varieties of apples were found to consist essentially of amyl esters of formic, acetic, and caproic acids, and "a considerable portion" of acetaldehyde (Power and Chesnut, 1920). Traces of methanol, ethanol, and possibly some free formic, acetic, and caproic acids were also found. From further investigations, Power and Chesnut (1922), Gane (1934) and Walls (1942) reported geraniol, ethylene and an amyl derivative. White (1950) isolated furfural, three aldehydes, and ten alcohols from McIntosh and Stayman Winesap apples by concentration and fractionation, but esters as such were not identified. Thompson (1951), Thompson and Huelin (1951), and Huelin (1952), in a series of articles, identified 6 acids, 4 alcohols, 2 aldehydes, and acetone in the volatiles produced by whole Granny Smith apples. Mehlitz and Gierschner (1961) described the extraction of aroma compounds from apple juice with isopentane. Strackenbrock (1961) studied the flavors of 8 different varieties of apples, and found 18 compounds.

EXPERIMENTAL

Preparation of volatile material from apple juice. Two tons of fresh McIntosh apples were milled and pressed, and the juice was filtered through cheesecloth. The apple juice volatiles were concentrated approximately tenfold by stripping in a rising film evaporator which had a 10-ft × 1½-in. steam-jacketed heating tube and a shell-type heat exchanger with 25-ft² surface area. Cooling water temperature was 13°C. This process yielded approximately 300 lb of a condensate having a good apple odor. The condensate was stored at −29°C until required for extraction. This frozen condensate was thawed and used as needed.

The neutral fraction was extracted from the condensate in 150-ml batches with ethyl chloride as described by Matthews *et al.* (1962). No attempt was made to eliminate the acidic fraction before extraction.

The acid fraction was prepared as follows:

1) Four liters of the apple juice condensate were adjusted to pH 8.1 by the addition of $CaCO_3$ solution.

2) The neutral fraction was removed by extraction with ethyl chloride (Matthews *et al.*, 1962). Most of this neutral fraction was removed by two extractions. This fraction was discarded and the remaining aqueous layer was adjusted to pH 1.0 with 18*N* H_2SO_4.

3) The acidic fraction was removed by extrac-

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tion with ethyl chloride and the ethyl chloride removed (Matthews *et al.*, 1962).

4) The acids thus obtained from the apple juice condensate were methylated by using the ether solution of diazomethane prepared from nitroso-methyl-urethan. (Organic Syntheses, 1943).

Four liters of juice condensate yielded sufficient extract for subsequent chromatography.

Separation and identification of volatiles. The volatiles were separated into 5 main fractions on an Aerograph A-90-P gas chromatograph equipped with a 6-ft × ¼-in. stainless-steel column and a Beckman fraction collector. Many types of solid and stationary-phase materials were tested before it was found that 30% by weight Carbowax-400 (polyethyleneglycol) on acid-washed 40–60-mesh firebrick C-22 gave adequate preliminary separation of the extract components. For the rough separation, 100-μl samples were used. Fig. 1 shows a typical separation when only a 5-μl sample was injected.

Further separation of the five fractions thus obtained from several 100-μl samples of the neutral components of apple juice condensate was carried out on the following three columns: 1) 6-ft × ¼-in. packed with 30% Carbowax-400 on firebrick C-22; 2) 10-ft × ¼-in. double column consisting of 5 ft of Tide (commercial detergent, alkylaryl sulfonate mixture) and 5 ft of 10% Carbowax-400 on firebrick C-22; 3) 10-ft × ¼-in. 20% silicone SE-30 on acid-washed 60–80-mesh Chromosorb W. Tide for the column packing was heated overnight in a drying oven at 110°C to evaporate the perfume and moisture. A screened sample of 40–60-mesh was packed into a column tube. The column was conditioned for 3 hrs at 200°C before being connected with the Carbowax-400 column. The dual column was used to separate a mixture of 2-methyl-

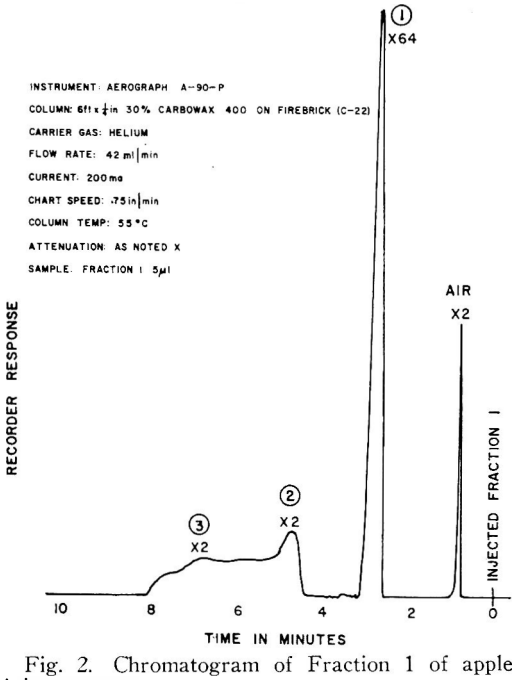

Fig. 2. Chromatogram of Fraction 1 of apple juice extract.

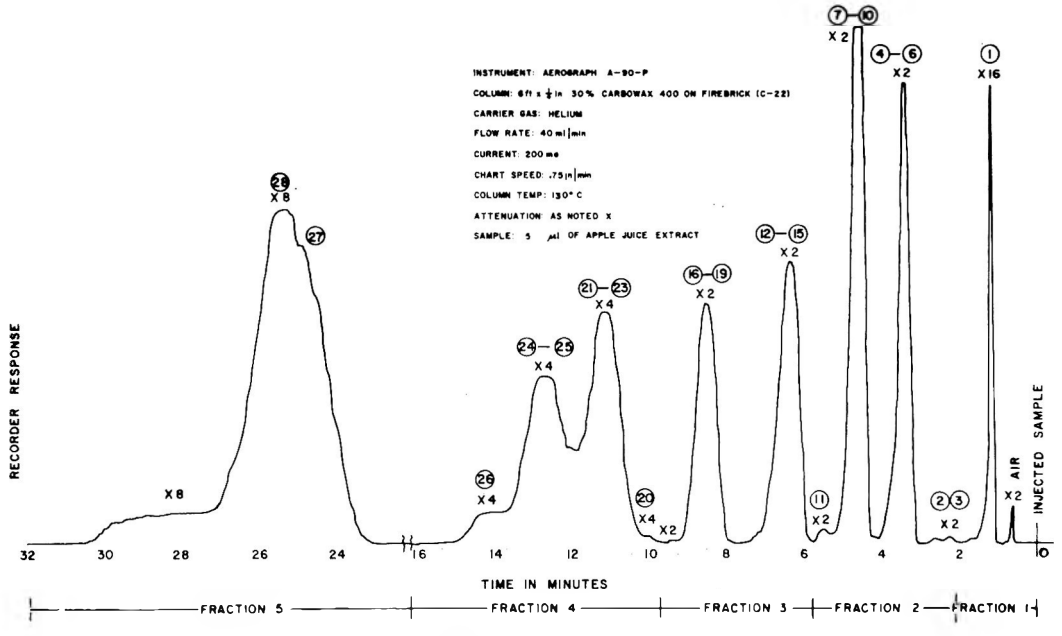

Fig. 1. Chromatogram of apple juice extract.

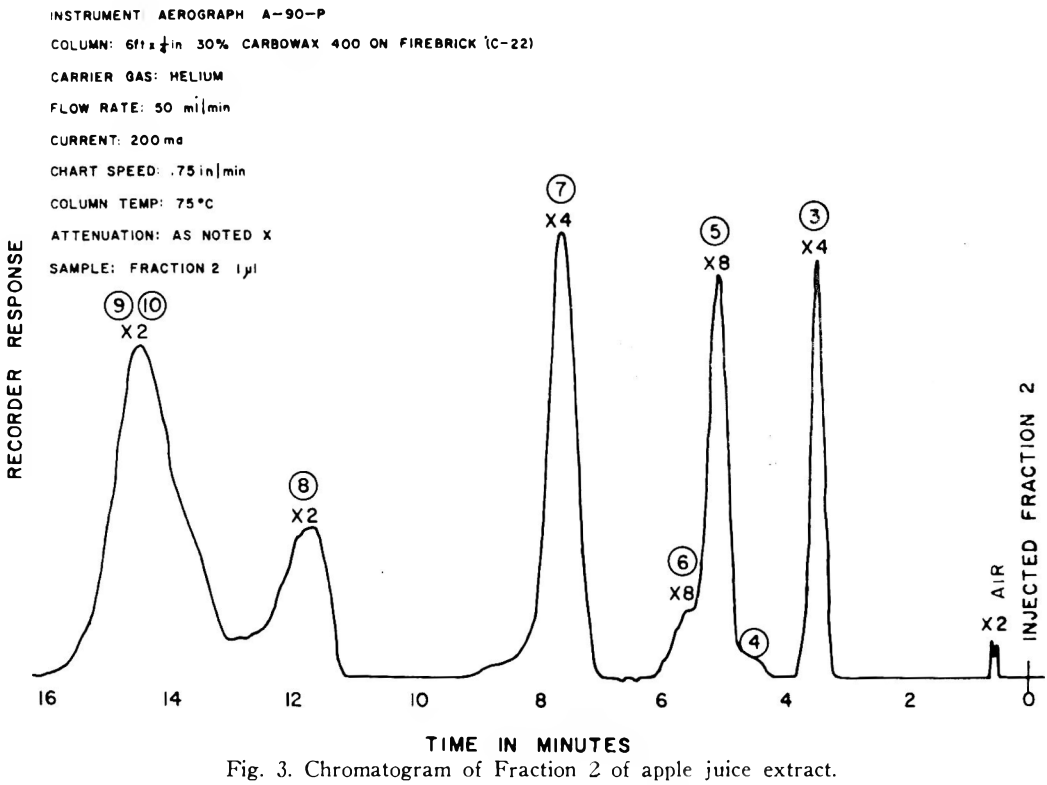

Fig. 3. Chromatogram of Fraction 2 of apple juice extract.

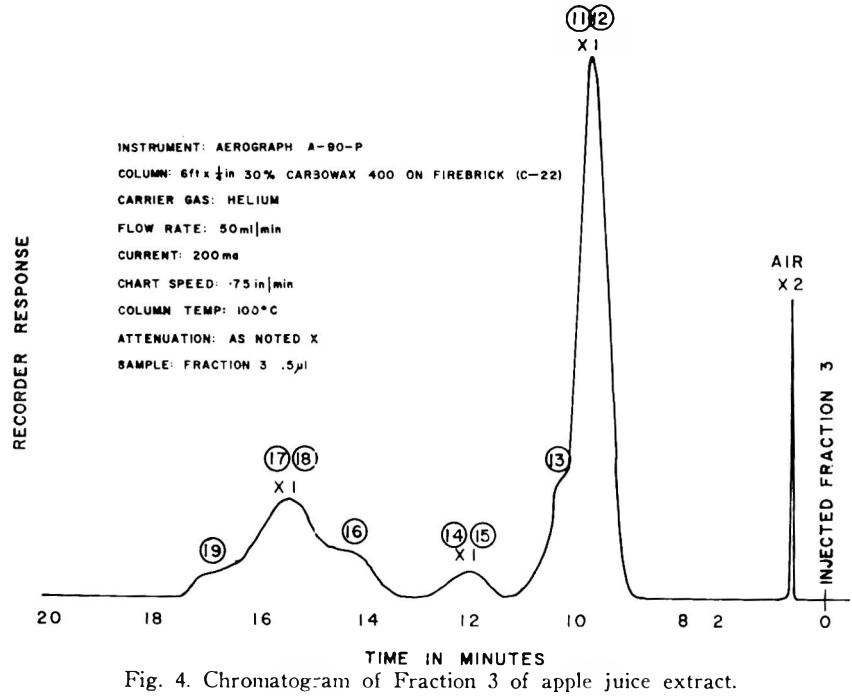

Fig. 4. Chromatogram of Fraction 3 of apple juice extract.

1-butanol and iso-pentanol. The 20% silicone SE-30 column was used for the separation of 2-hexene-1-al (2-hexenal).

When at least 50 µl of each of the five major fractions shown in Fig. 1 had been collected, fractions 1, 2, 3, and 4 were separated under the con-

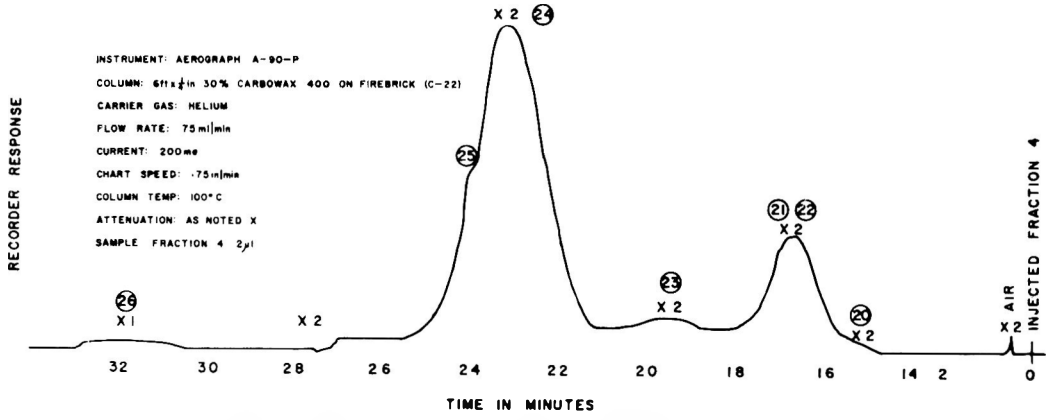

Fig. 5. Chromatogram of Fraction 4 of apple juice extract.

ditions shown in Figs. 2, 3, 4, and 5, and their components collected. Retention volumes (see Tables 2 and 3) of the individual components relative to acetone were measured. Additional evidence for the identity and purity of each component collected was obtained by rechromatography on an Aerograph A-600-B equipped with a flame ionization detector and a 10-ft × ⅛-in. stainless-steel column packed with 10% Ucon non-polar on acid-washed 60–80-mesh Chromosorb W (Fig. 6).

The relative retention volumes for fraction 5 obtained with Carbowax-400 were open to doubt because of unsatisfactory separation of the individual components. Therefore, further retention data were obtained for this fraction with a flame ionization detector and a 5-ft × ⅛-in. stainless-steel column packed with 5% Silicone SE-30 on acid-washed 60–80-mesh Chromosorb W.

The ether solution of the methylated sample prepared from the acidic fraction was studied with the Aerograph A-600-B equipped with a hydrogen flame ionization detector. Columns were of two types: 10-ft × ⅛-in. 10% Carbowax-400 on acid-washed 60–80-mesh Chromosorb W; and 5-ft × ⅛-in. 10% diethyleneglycol succinate (DEGS) on acid-washed 60–80-mesh Chromosorb W (Fig. 7).

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

Infrared spectroscopy. Infrared spectra of selected fractions (see Table 1) were determined after purification of each fraction (5 µl) by rechromatography showed only one well defined component. Spectra were determined on a Perkin-Elmer model 137 Infracord spectrophotometer. When sufficient material was available the thin-film technique was used with the material layered between two NaCl discs. When sample size was seriously restricted, a micro-cavity cell (0.2 mm) was used to determine the spectra in spectro-grade CCl_4.

RESULTS AND DISCUSSION

Tables 2, 3, and 4 give the relative reten-

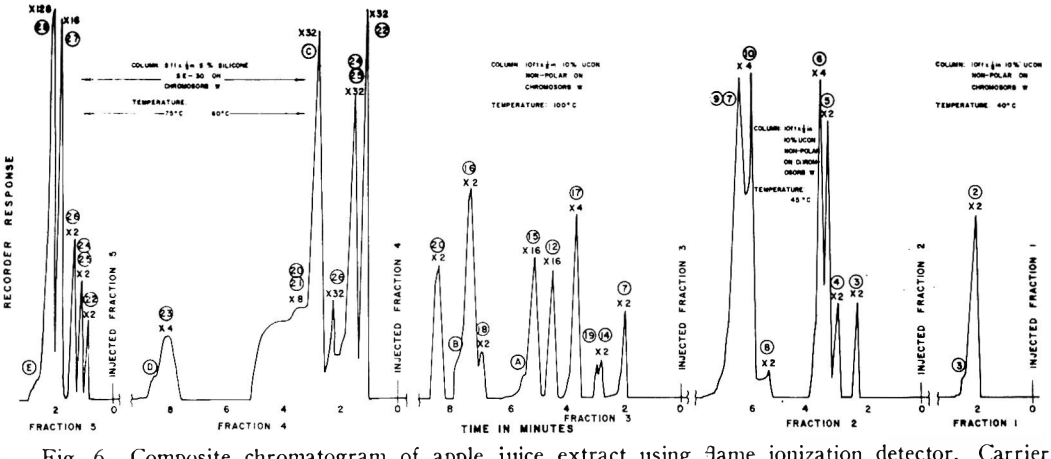

Fig. 6. Composite chromatogram of apple juice extract using flame ionization detector. Carrier gas, nitrogen; flow rate 20 ml/min. Attenuation as noted.

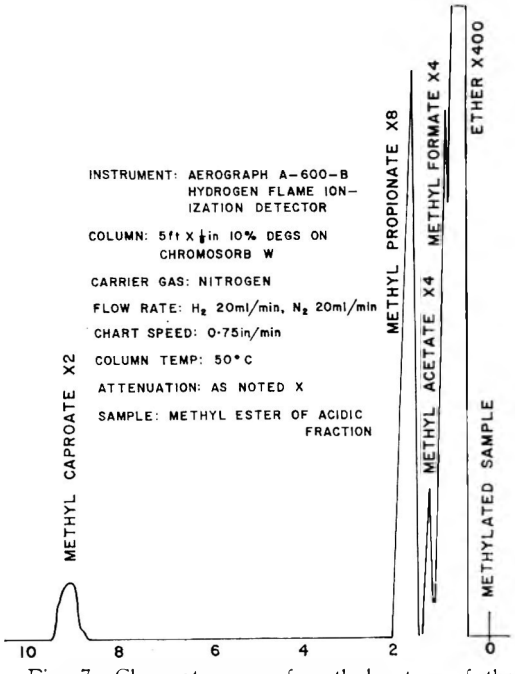

Fig. 7. Chromatogram of methyl esters of the acidic fraction of apple juice volatiles.

tion volumes of the volatile components of apple juice and authentic compounds.

Acetone was used as the reference compound for the neutral fraction, and methyl propionate for the acidic fraction.

Peaks 1 and 11 and peaks A, B, C, D, and E (Figs. 1, 2, 6) were not identified. It is hoped that work still in progress will determine at least some of these.

The boiling point of the compound represented by peak 1 (Fig. 1) was quite low, and two chromatograms contained this peak. It is not ethyl chloride.

iso-Butyl propionate (peak 13, Fig. 4) was tentatively identified by means of only one column. This peak did not show up when the sample was chromatographed with a flame ionization detector using a Ucon nonpolar column. Separation was achieved by using a thermistor detector and Carbowax-400.

Peaks 17 and 18 (Fig. 4), *iso*-butanol and *iso*-amyl acetate, could not be separated with the 6-ft × ¼-in. 30% Carbowax-400 column, but they were separated very well with the 10-ft × ¼-in. double column consisting of Tide and Carbowax-400 in series.

Peak 19 (Table 2) is shown as an isomer

Table 1. Volatile components found in apple juice.

	Method of identification[a]
Alcohols	
Methanol	RV
Ethanol	RV
iso-Propanol	RV
n-Propanol	RV
iso-Butanol	RV-I
n-Butanol	RV-I
2-methyl-1-Butanol	RV-I
iso-Pentanol	RV-I
sec-Pentanol	RV
n-Pentanol	RV-I
n-Hexanol	RV-I
Aldehydes	
Acetaldehyde	RV
Propionaldehyde	RV
Caproaldehyde	RV-I
2-Hexenal	RV-I
Esters	
Methyl formate	RV
Ethyl formate	RV
Ethyl acetate	RV
n-Butyl acetate	RV
iso-Amyl acetate	RV-I
n-Amyl acetate	RV
iso-Butyl propionate	RV
Ethyl-*n*-Butyrate	RV-I
Methyl-*n*-caproate	RV
Ethyl-*n*-caproate	RV-I
Ketone	
Acetone	RV
Acids	
Formic	RV
Acetic	RV
n-Propionic	RV
n-Caproic	RV

[a] RV, retention volumes; I, infrared.

of *sec*-pentanol. The relative retention volume corresponds to a peak obtained with an isomer of commercial *sec*-pentanol.

An anomaly encountered occasionally is the inability of some columns to separate a mixture of two compounds that have considerably different retention volumes when chromatographed singly. This situation is demonstrated by the inability to separate 2-methyl-1-butanol (*act*-pentanol) and 3-methyl-1-butanol (isopentanol) on a silicone SE-30 column or Ucon nonpolar column (Table 3 and Fig. 5). Porcaro and Johnston

Table 2. Relative retention volumes of apple juice components and authentic compounds.

Peak no.	Name	Column A Relative retention volumes		Column B Relative retention volumes	
		Standard	Sample	Standard	Sample
2	Acetaldehyde	0.45	0.46	0.55	0.57
3	Methyl formate	0.64	0.64	0.63	0.63
4	Propionaldehyde	0.75	0.75	0.79	0.79
5	Ethyl formate	0.86	0.87	0.95	0.93
6	Acetone	1.00	1.00	1.00	1.00
7	Ethyl acetate	1.30	1.31	1.77	1.77
8	Methanol	1.96	1.94	1.49	1.51
9	*iso*-propanol	2.27	2.42	1.75	1.75
10	Ethanol	2.37	2.42	1.68	1.68
12	Ethyl-*n*-butyrate	3.00	3.05	3.00	3.00
13	*iso*-Butyl propionate	3.29	3.30	4.11	.....
14	*n*-Propanol	3.85	3.82	1.79	1.79
15	*n*-Butyl acetate	3.75	3.82	3.45	3.39
16	Caproaldehyde	4.60	4.61	4.88	4.88
17	*iso*-Butanol	4.84	4.90	2.45	2.40
18	*iso*-Amyl acetate	4.90	4.90	4.66	4.66
19	Isomer of *sec*-pentanol	5.20	5.30	2.00	1.95

Column A: 6-ft × ¼-in. 30% Carbowax-400 on firebrick C-22 acid-washed, mesh 40–60, Aerograph A-90-P, thermistor detector.
Column B: 10-ft × ⅛-in. 10% Ucon-nonpolar on Chromosorb W acid-washed mesh 60–80, Aerograph A-600-B, hydrogen flame ionization detector.
Relative retention volumes related to acetone.

(1961) reported that these two compounds could not be separated with Carbowax-20M on Chromosorb W, but that Tide, without an added stationary phase, effected separation. We examined the separation of *n*-, *iso*-, and *act*-pentanol by using three columns: Tide, Carbowax-400, and Sorapon (a commercial antifoam alkylaryl sulfonate); and the dual column consisting of Tide and Carbowax-400 (Table 5). We found that, as well as 2-methyl-1-butanol and 3-methyl-1-butanol, other alcohols could also be separated by the double column.

Peak 27 is shown as 2 hexenal (2 hexene-1-al). This compound was partially identified by infrared and boiling point (150–

Table 3. Relative retention volumes of apple juice components and authentic compounds.

Peak no.	Name	Column A Relative retention volumes		Column C Relative retention volumes		Column D Relative retention volumes	
		Standard	Sample	Standard	Sample	Standard	Sample
20	*n*-Amyl acetate	6.07	6.10	9.15	9.32	4.03	4.00
21	Methyl caproate	6.51	6.68	9.50	9.32	4.50	4.50
22	*n*-Butanol	6.70	6.68	2.33	2.33	2.33	2.33
23	Ethyl caproate	7.66	7.70	18.08	18.02	6.68	6.80
24	2-Methyl-1-Butanol	9.10	9.19	3.16	3.33	3.33	3.33
25	*iso*-Pentanol	9.58	9.58	3.00	3.33	3.33	3.33
26	*n*-Pentanol	12.75	12.65	4.00	4.16	4.03	4.00
27	2-Hexenal	.....	12.80	.....	5.75	.....	2.00
28	*n*-Hexanol	12.79	12.80	7.00	7.00	2.80	2.80

Column A: 6-ft × ¼-in. 30% Carbowax-400 on firebrick C-22 acid-washed, mesh 40–60. Aerograph A-90-P, thermistor detector.
Column C: 5-ft × ⅛-in. 5% silicone SE-30 on Chromosorb W acid-washed, mesh 60–80. Aerograph A-600-B, hydrogen flame ionization detector.
Column D: 8-ft × ⅛-in. 2% Ucon-nonpolar on Chromosorb W acid-washed, mesh 60–80. Aerograph A-600-B, hydrogen flame ionization detector.
Relative retention volumes related to acetone.

Table 4. Relative retention volumes of the methyl esters of the acidic fraction of apple juice and authentic compounds.

	Column E		Column F	
	Relative retention		Relative retention	
Compounds	Standard	Sample	Standard	Sample
Formate	0.62	0.62	0.52	0.52
Acetate	0.77	0.77	0.72	0.72
n-Propionate	1.00	1.00	1.00	1.00
n-Caproate	3.40	3.38	4.95	4.92

Retention volumes relative to methyl propionate.
Column E: 10-ft × ⅛-in. 10% Carbowax-400 on Chromosorb W, acid-washed, mesh 60–80. Aerograph A-600-B, hydrogen flame ionization detector.
Column F: 5-ft × ⅛-in. 10% DEGS on Chromosorb W, acid-washed, mesh 60–80. Aerograph A-600-B, hydrogen flame ionization detector.

Table 5. Comparison of relative retention volumes of alcohols measured by three different columns.

	Column G	Column H	Column I
Methanol	5.75	1.64	3.17
Ethanol	2.48	1.78	1.66
iso-Propanol	1.20	1.90	1.22
n-Propanol	2.96	2.46	1.88
iso-Butanol	1.68	4.46	1.75
n-Butanol	3.52	6.42	2.58
2-Methyl-1-butanol	2.60	9.30	2.70
iso-Pentanol	3.35	10.00	3.10
n-Pentanol	4.16	12.82	4.00

Retention volumes relate to acetone. These retention volumes were measured by Aerograph A-90-P, thermistor detector.
Column G: 10-ft × ¼-in. Tide, mesh 40–60, 130°C, He 50 ml/min.
Column H: 10-ft × ¼-in. 10% Carbowax-400 and 5% sorapon on Chromosorb W, acid-washed, mesh 60–80, 75°C, He 75 ml/min.
Column I: 10-ft × ¼-in. dual column, 130°C, He 50 ml/min. 1st column, 5-ft × ¼-in. Tide, mesh 40–60. 2nd column, 5-ft × ¼-in. 10% Carbowax-400 on Chromosorb W, acid-washed, mesh 60–80.

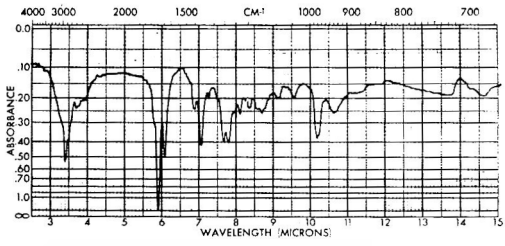

Fig. 8. Infrared spectrum of Peak 27 (2-hexenal). About 2% CCl_4 solution in micro-cavity cell.

151°C) derived from the relationship between retention volumes and boiling point. The infrared spectrum of this fraction, separated by using a 10-ft. × ¼-in. 20% silicone SE-30 on Chromosorb W column, is given in Fig. 8, and shows characteristic absorption bands as follows: olefinic C = C, 1645 cm^{-1}; CH_3 and CH_2, 1456 and 1379 cm^{-1}; conjugated CHO, 1700 cm^{-1}; and C—H stretching, 2890 cm^{-1}. Suggesting a structure of $CH_3CH_2CH_2CH = CHCHO$ (2-hexenal). White (1950) found this compound in apple juice. Takei and Huelin (1933) and Takei *et al.* (1938) also reported that 2-hexenal was in part responsible for the "green" odor in tea.

These data show thirty compounds at least partially identified, and seven completely unknown. The neutral components of apple juice volatiles are present in greater numbers than the eighteen that were found in apple wine (Matthews *et al.*, 1962), and fatty acids are fewer in apple juice than in apple wine (Sugisawa *et al.*, 1962). This apparent decrease during fermentation may be explained in one or more of the following three ways: 1) loss of volatiles by the purg-

Table 6. Tabulation of alcohols, esters, and acidic ester components found in apple juice.

	Acid ester component				
	Alcohol	Formic	Acetic	Propionic	Butyric	Caproic
Methanol	+	+				+
Ethanol	+	+	+		+	+
iso-Propanol	+					
n-Propanol	+					
iso-Butanol	+			+(?)		
n-Butanol	+		+			
2-Methyl-1-butanol	+					
iso-Pentanol	+		+			
sec-Pentanol	+					
n-Pentanol	+		+			
n-Hexanol	+					

ing action of carbon dioxide; 2) metabolism of some compounds by the fermenting yeasts; 3) inefficient separation of some volatiles due to the large quantity of ethanol in wine or solvent selectivity to some compounds. At the present stage of this work it is not possible to determine which of these processes is active. However, future work will follow the volatiles during fermentation and perhaps resolve this problem.

Eleven alcohols have been identified, several of which occur without corresponding esters. However, all ten of the esters present are accompanied by their homologous alcohols. Butyric acid occurs in one ester, but apparently not as the free acid (Table 6).

ACKNOWLEDGMENT

The authors are grateful to Sun-Rype Products Limited for the apples used. Thanks are also due to the Department of Chemistry, University of British Columbia, Vancouver, for use of the infrared spectrophotometer, and Dr. J. E. Bloor, of the British Columbia Research Council, for help in determining infrared spectra.

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The Precursors of Chocolate Aroma: A Comparative Study of Fermented and Unfermented Cocoa Beans

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(Manuscript received January 30, 1964)

SUMMARY

The precursors of chocolate aroma were concentrated, in a fraction amounting to some 5% of the fermented shell-free cocoa bean, by solvent extraction and dialysis. The concentrate, which was soluble in both methanol and water, comprised sugars, amino acids, and flavonoids, and produced chocolate aroma when heated. Examination of unfermented cocoa bean revealed significant differences in these components prior to fermentation.

INTRODUCTION

Cocoa beans develop the characteristic primary aroma of chocolate only after they have been roasted. Unroasted beans must therefore contain substances, already styled "chocolate aroma precursors," which give rise to the primary smell of chocolate when heated together. Unfermented cocoa beans produce a smell resembling broad beans when roasted (Knapp, 1937). Fermentation is thus an essential stage of cocoa processing which is accompanied by the development of the aroma precursors. The literature has revealed useful if sometimes equivocal information on the possible nature and properties of the aroma precursors. A review has already been given in a recent publication (Rohan, 1963) showing that flavonoids, purines, and amino acids have been considered as playing some role in the development of chocolate aroma. The same publication described the isolation from fermented cocoa beans of a fraction, comprising flavonoids, sugars, and amino acids, which gave the characteristic primary smell of chocolate when heated. The present communication is an extension of that work, in which fermented and unfermented cocoa beans have been examined comparatively in an attempt to determine the relative importance of the three groups of compounds into which the aroma precursors have already been concentrated. Commercial samples of Ghana cocoa beans were used throughout this work. Ghana cocoa, which is of the amelonado variety, is normally fermented in heaps for 4–6 days, after which it is dried in the sun. It has the distinct advantage of being the most uniform commercial cocoa available, and is thus admirably suited for such an investigation as that described in this publication.

EXPERIMENTAL

Fermented and unfermented cocoa beans were shelled and finely ground. The powdered materials (1 kg each) were extracted with warm (50°C) 80% aqueous methanol (1 L each), and the two extracts fractionated as described in a previous publication (Rohan, 1963) and outlined in Fig. 1. Only fractions B, D, and F from fermented cocoa beans produced chocolate aroma when heated at 140°C for 8 min. The ultimate concentrates (fractions F) were each shown to comprise essentially flavonoids, amino acids, and sugars. These fractions were isolated by precipitation of the flavonoids, from aqueous solutions of the fractions F, with saturated lead acetate, and separation of the flavonoid-free residues into amino acid and sugar fractions on a cation-exchange resin (Amberlite IR.120.H). The three main groups thus isolated from fermented and unfermented cocoa beans were examined in further detail. The carbohydrate fractions were chromatographed in Whatman No. 1 paper with a variety of irrigating solvents, and the individual sugars were located on the chromatograms with chromogenic reagents. The amino acids were quantitatively and qualitatively analyzed on a Beckman/Spinco amino acid analyzer. The flavonoid residues were studied in the untreated fractions F, because of their known instability.

RESULTS AND DISCUSSION

When finely ground shell-free ghana cocoa beans were fractionated according to the

system outlined in Fig. 1, fermented beans yielded fraction F in approximately 5% yield, and unfermented beans yielded fraction F in approximately 12% yield. Each of these fractions consisted essentially of sugars, amino acids, and flavonoids. The

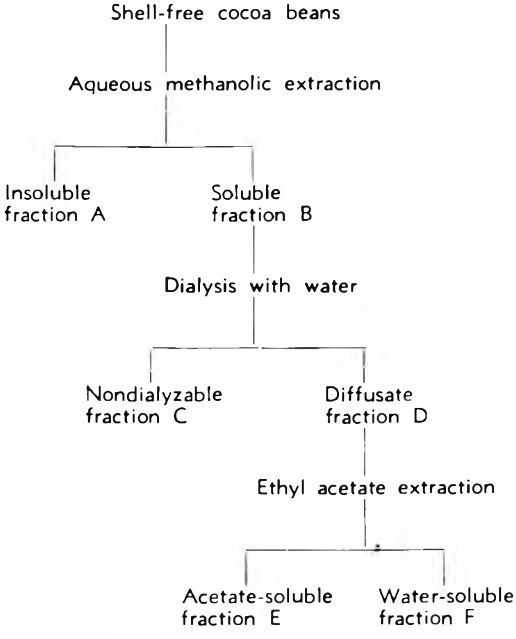

Fig. 1. Fractionating procedure for cocoa beans.

fraction from fermented beans produced chocolate aroma when heated at 140°C for eight minutes, whereas the corresponding fraction from unfermented beans did not. Table 1 gives the relative compositions of the fractions F from fermented and unfermented beans.

Table 1. Approximate relative compositions of fractions F from fermented and unfermented cocoa beans.

Fraction	% of fraction F	
	Fermented	Unfermented
Flavonoid	30	27
Carbohydrate	35	56
Amino acid	25	7

Examination of component groups of fractions F. *Carbohydrate fractions.* Chromatography on paper showed that whereas the sugars in fermented cocoa beans comprised mainly glucose and fructose, unfermented cocoa beans contained only sucrose (Table 2). Bailey and his colleagues (1962) detected isovaleraldehyde, isobuteraldehyde, *n*-buteraldehyde, propionaldehyde, and acetaldehyde among the volatile components of chocolate aroma, and postulated their formation from amino acids by means of a Strecker degradation. Glucose has been shown to react with α-amino acids to yield aldehydes with one carbon atom less than the amino acids used (Schonberg and Moubacher, 1952), and Frankel and Katchalsky (1937) have demonstrated that glucose reacts with amino acids in the related nonenzymatic browning (Maillard) reaction whereas sucrose does not. There is evidence that fructose can also participate in the Maillard reaction, and the absence of both fructose and glucose in unfermented cocoa beans might, therefore, be significant.

Amino acid fractions. The two amino acid residues were anlyzed on a Beckman/Spinco analyzer. The results (Table 3) confirm that fermentation is accompanied by an increase in the free amino acid concentration (DeWitt, 1957). They do not allow any conclusions to be made on the possible importance of the individual acids as chocolate aroma precursors.

Flavonoid fractions. The flavonoid fractions contained flavylogens (Swain, 1959) as evidenced by the production of cyanidin on boiling with acid. These compounds are known to be unstable and were precipitated from each of the fractions F by diluting their methanolic solutions with diethyl ether. The resultant precipitates were examined by chromatography on Whatman No. 1 paper, and the comparative results are given in Table 4.

CONCLUSIONS

Results show that the aqueous methanolic-soluble components of both fermented and unfermented cocoa beans comprise amino acids, sugars, and flavonoids. The extract from fermented cocoa produced chocolate aroma when heated but that form unfermented cocoa did not. It is probable that the carbohydrate fraction in unfermented cocoa (sucrose) is the precursor of the corresponding fraction in the fermented material (glucose and fructose). It is likely, also, that the simple (more

Table 2. Rg[a] values of sugars in fermented and unfermented cocoa beans.

Spot	2-PrAq[b]	EtAcPy[c]	PrEtAc[d]	2-PrBu[e]	iBuA[f]	EtAcA[g]	2-PrPy[h]	BuA[i]	Spot intensity
Fermented beans									
Sugars revealed with aniline/phosphoric acid									
1	100	100	100	100	100	100	100	100	Strong
2	107	....	114	118	120	144	108	138	Strong
3	46	29	32	40	45	....	....	41	Weak
4	22	9	12	16	22	....	....	14	Weak
fructose	107	116	114	113	124	150	105	135	
Sugars revealed with naphthoresorcinol/phosphoric acid									
2	108	122	116	106	108	133	105	126	Strong
3	45	35	30	34	37	....	....	....	Weak
4	21	18	12	16	16	30	24	12	Weak
5	81	73	78	83	74	45	92	....	Weak
sucrose	87	68	77	79	78	45	95	80	
Unfermented beans									
Sugars revealed with aniline/phosphoric acid									
1	89	90	74	81	78	50	93	77	Strong
2	42	50	29	35	36		57	37	Weak
3	15	24	12	13	17	12	27	17	Weak
fructose	107	107	113	108	113	144	104	110	
Sugars revealed with naphthoresorcinol/phosphoric acid									
1	88	90	74	85	86	53	94	68	Strong
2	42		28	34	32	22	57	32	Weak
3	15	24	11	14	14	12	28	11	Weak
sucrose	88	90	76	82	79	53	94	75	

[a] $Rg = \frac{\text{distance travelled by substance from origin}}{\text{distance travelled by glucose from origin}} \times 100$

[b] 2-propanol–water, 160 : 40 v/v.
[c] ethyl acetate–pyridine–water, 120 : 50 : 40 v/v.
[d] propanol–ethyl acetate–water, 140 : 20 : 40 v/v.
[e] 2-propanol–butanol–water, 140 : 20 : 20.
[f] isobutanol–acetic acid–water, 120 : 30 : 50 v/v.
[g] ethyl acetate–acetic acid–water, 140 : 30 : 30 v/v.
[h] 2-propanol–pyridine–water, 120 : 40 : 40, v/v.
[i] butanol–acetic acid–water, 60 : 15 : 25 v/v.

mobile) flavonoid compounds of unfermented cocoa are the precursors of the more complex substances in the fermented bean (*cf.* Forsyth, 1952). The substantial increase in free amino acids after fermentation suggests that their precursors are to be found in the insoluble protein fraction of the unfermented cocoa. Although the relative importance of the three principal precursor groups is yet to be settled, their nature suggests that the operative reaction in the development of chocolate aroma might be a Strecker degradation of the amino acid fraction. This could occur through the agency of the flavonoids (Jackson and Kendall, 1949; Trautner and Roberts, 1950; Isherwood and Niavis, 1956). Alternatively glucose and fructose

Table 3. Amino acids of fremented and unfermented cocoa beans (fractions F).

Amino acid	g amino acid/100 g fraction F	
	Fermented	Unfermented
Lysine	0.56	0.08
Histidine	0.036	0.08
Arginine	0.35	0.08
Threonine	0.84	0.14
Serine	1.99	0.88
Glutamic acid	1.77	1.02
Proline	1.97	0.72
Glycine	0.35	0.09
Alanine	3.61	1.04
Valine	2.60	0.57
isoLeucine	1.68	0.56
Leucine	4.75	0.45
Tyrosine	1.27	0.57
Phenylalanine	3.36	0.56

Table 4. R_f values of vanillin reactive components of fermented and unfermented cocoa beans (fractions F).

Solvent	Rf × 100		Vanillin color intensity	
	Fermented	Unfermented	Fermented	Unfermented
2% acetic acid	0	0	intense	none
	0 – 50	0 – 55	very weak	intense
		80		weak
Forestal solvent [a]	0	0	intense	weak
	0 – 50	0 – 50	weak	weak
sec Bu [b]	0	0	intense	none
	13	5 – 30	weak	strong
	20		weak	
BuAc [c]	0	0	intense	none
	0 – 17	0 – 5	weak	strong

[a] Acetic acid–HCl–water, 30 : 3 : 10 v/v.
[b] Sec-butanol saturated with water.
[c] Butanol–acetic acid–water, 60 : 15 : 25 v/v.

could be the other reactants, and their absence in unfermented cocoa could then be significant. Use is now being made of gas chromatography to study the volatile materials produced when extracts of fermented and unfermented cocoa beans are heated, and it is hoped that this will throw more light on the basic reaction involved.

ACKNOWLEDGMENT

The author is indebted to Dr. M. J. Crumpton and Mr. J. M. Wilkinson, of the Wright-Flemming Institute of Microbiology, for amino acid analyses.

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The Precursors of Chocolate Aroma: A Study of the Flavonoids and Phenolic Acids

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(Manuscript received January 30, 1964)

SUMMARY

Flavonoids and phenolic acids were identified in the ethyl-acetate-soluble fraction of the precursors of chocolate aroma. In addition to previously identified flavanols and flavylogens (leucoanthocyanins), two flavonols (quercetin and quercetrin) and three phenolic acids (*p*-coumaric, caffeic, and chlorogenic) were found. The ethyl-acetate-soluble fraction of the aroma precursors does not appear to be essential to production of the primary characteristic aroma of chocodate.

INTRODUCTION

The flavonoids and phenolic acids described in this communication were removed from a methanol extract of fermented Ghana cocoa beans, with ethyl acetate, during isolation of the precursors of chocolate aroma (Rohan, 1963). The flavonols of Ghana cocoa have already been examined both quantitatively and qualitatively (Rohan, 1958), but the minor phenolic constituents have not yet been given any serious attention. Griffiths (1958) described separation and characterization of some of these compounds in Trinidad cocoa, and the results were slightly different from those described below. The present work is part of an investigation of the nature of the primary characteristic aroma of chocolate.

EXPERIMENTAL

Identification of flavonoids and phenolic acids. Except where otherwise stated, Whatman No. 1 paper was used for all chromatographic work. Chromatograms were examined in UV light before and after fuming with ammonia, and the following chromogenic reagents were also used to aid in identification.

Barton's reagent (Barton *et al.*, 1952). A 1% solution of ferric chloride, mixed just before use with a 1% solution of potassium ferricyanide, gave a blue color with catechol/pyrogallol-type polyphenols.

Vanillin reagent. A 1% ethanolic solution of vanillin, mixed with an equal volume of concentrated hydrochloric acid immediately before use, gave characteristic pink colors with flavonoid compounds containing phloroglucinol nuclei.

Toluene p-*sulfonic acid* (Roux, 1957). A 1% solution of the reagent in ethanol produced a red coloration with flavylogens (Swain, 1959) not hydroxylated in the 5-position, when chromatograms were heated after spraying; and a yellow-red color, on more prolonged heating, with flavylogens hydroxylated in the 5-position.

bis-Diazotized benzidine (Pictet and Brandenberger, 1960). This reagent gave characteristic colours with phenolic acids.

Diazotized p-*nitraniline* (Swain, 1953). This reagent was particularly useful for characterization of phenolic acids.

Hopfner reagent (Walker, 1962). Equal volumes of a 5% solution of acetic acid and a 5% solution of sodium nitrite, mixed just before use, gave characteristic colors with phenolic acids.

Aluminum chloride (Gage and Wender, 1950). A 5% ethanolic solution of the reagent caused a bathochromic shift in the absorption of flavonols which resulted in characteristic color changes in UV light.

Sodium carbonate. A 20% aqueous solution of the reagent gave characteristic colors with various flavonoid compounds.

Extraction and separation of flavonoids and phenolic acids. Finely ground shell-free commercial Ghana cocoa beans were used for this investigation. The raw material was harvested and cured according to standard practice. The powder (100 g) was warmed with methanol (50°C) containing 20% water (250 ml). After removal of the insoluble residue by filtration, the solution was freed from organic solvent, and the aqueous residue was dialyzed against distilled water with the use of Visking cellulose casing. The diffusate was concentrated and exhaustively extracted with cold ethyl acetate. The solvent extract was evaporated to dryness, and the residue fractionated as described in the following paragraphs.

Acetone-insoluble fraction. This was obtained by triturating the ethyl-acetate-soluble material

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with cold acetone, filtering, and washing the residue with acetone. Chromatography on paper with 2% aqueous acetic acid and butanol–acetic acid–water (60:15:25 v/v) used as irrigating solvents, was employed in examination of this fraction.

Chloroform-insoluble fraction. The acetone solution was concentrated and diluted with four volumes of chloroform. The resultant precipitate was filtered and reprecipitated from its acetone solution with chloroform. This was repeated a third time, and the combined filtrates evaporated to dryness. The final chloroform precipitate, which contained a high concentration of vanillin-reactive material, was examined by two-way chromatography on paper with 2% acetic acid used for the first dimension and butanol–acetic acid–water (60:15:25 v/v) for the second.

Flavonol fraction. The acetone/chloroform-soluble material was dissolved in 10 ml water, carefully neutralized with dilute sodium bicarbonate solution (pH =9) and introduced into the first tube of a Craig counter-current separator. After 30 fundamental transfers with water-saturated ether as the mobile phase, the upper, ethereal, layer was removed from each tube, concentrated, and examined by paper chromatography. A 2% solution of acetic acid was used as the irrigating solvent. The dried chromatograms were examined in UV light, and the fractions of similar composition were combined. The bulked fractions were then resolved into their components by two-way preparative chromatography on Whatman No. 3MM paper with butanol–acetic acid–water used as the first solvent, and 2% acetic acid as the second. Individual flavonols were isolated by cutting from the dried paper, after locating in UV light, and eluting with methanol. Two components were thus obtained whose chromatographic behavior was studied in different solvent systems, including butanol–acetic acid–water, Forestal solvent (Bate-Smith, 1954) and 15% acetic acid.

Phenolic acid fraction. The neutral solution remaining in the first tube of the Craig counter-current separator after separation of the flavonols, was shaken with three equal volumes of ethyl acetate, made acid with 0.1*N* HCl, and extracted with diethyl ether. The ethereal extract was evaporated to dryness, yielding a yellow/brown-colored residue which was chromatographed on paper with a number of different irrigating solvents including 2% acetic acid, benzene–acetic acid–water (60:70:30 v/v), ethyl acetate saturated with water, butanol–acetic acid–water (60:15:25 v/v), 10% acetic acid, and Forestal solvent. The phenolic acids were located by examining the chromatograms in UV light before and after fuming with ammonia, and by treating with various chromogenic reagents including diazotized *p*-nitroaniline, Hopfner reagent, ethanolic aluminum chloride, and bis-diazotized benzidine.

RESULTS

Acetone-insoluble fraction. This constituted less than 10% of the original material. Two-way paper chromatography demonstrated the presence of unchanged anthocyanins and flavylogens, all of which have already been fully described (Rohan, 1958).

Table 1. Components of chloroform-insoluble fraction.

Component	$R_f \times 100$		Color with reagents		
	2% acetic	BuAc[a]	UV	vanillin	PTS[b]
Flavylogen 1	55	45	dark	red	yellow/red
Flavylogen 2	35	52	dark	red	yellow/red
Flavylogen 3	38	43	dark	red	yellow/red
Flavylogen 4	27	41	dark	red	yellow/red

[a] Butanol–acetic acid–water, 60:15:25 v/v.
[b] Toluene *p*-sulfonic acid; 1% ethanolic solution.

Table 2. Components of flavonol fraction.

Component	$R_f \times 100$[a]				Color with reagents[b]				
	15% acetic acid	H_2O	BuAc	Forestal	uv	UV — NH_3	Benzidine	Na_2CO_3	$AlCl_3$ + NH_3
Quercetin	–(–)	0(0[c])	61(64[c])	39(41[d])	Y	Y	R-O	O	Y-G
Quercetin 3-monoglucoside	27(37[b])	7(8[c])	64(58[c])	–(–)	Y-G	Y-G	O-B	Y	Y-G

[a] Present study (literature figure).
[b] Y, yellow; G, green; R, red; O, orange; B, brown.
[c] Lederer (1960).
[d] Bate-Smith (1962).

Chloroform-insoluble fraction. This material, when heated with concentrated hydrochloric acid and amyl alcohol, imparted a red color to the alcohol layer. Chromatography of the red alcoholic solution with Forestal solution gave one red-colored spot (R_f, 0.5) which turned blue when the paper was fumed with ammonia. This corresponds with cyanidin (Bate-Smith, 1954) and indicates the presence of flavylogens (leucoanthocyanins) in this fraction. Two-way paper chromatography confirmed the presence of the principal flavylogens already found in Ghana cocoa (Table 1).

Flavonol fraction. The five leading tubes of the Craig counter-current separator contained quercetin, which was identified by its R_f values in different solvent systems and by its color reactions with various chromogenic sprays (Table 2). Confirmation was obtained by co-chromatography with authentic quercetin, from which it did not separate in any of the solvent systems described. Tubes 20 to 30, the least mobile fraction, contained a flavonol glycoside which was isolated by two-way preparative chromatography on Whatman No. 3MM paper with butanol-acetic acid-water (60:15:25 v/v) used for the first dimension and 2% acetic acid for the second. The glycoside whose chromatographic properties are shown in Table 2 was hydrolyzed by heating with 2*N* HCl for 1 hr on a steam bath. The cooled hydrolysate was extracted with ether, and the ethereal solution chromatographed, after suitable concentration, with Forestal solvent. One yellow spot was observed in visible light and intensified in UV light (R_f 0.36). This did not separate from authentic quercetin when co-chromatographed. The aqueous residue from the ether extraction was freed from acid on a short column of anion-exchange resin (Amberlite 1R4B.OH), concentrated and chromatographed on paper with six different solvent systems (*cf.* Rohan, 1963). Treatment of the chromatograms with aniline phosphate revealed only one sugar with R_f values identical with those of glucose. The quercetin glycoside has been tentatively identified as quercetin 3-monoglucoside (iso quercetrin).

Acid fraction. The acids were chromatographed on paper with various irrigating solvents and located on the chromatograms with UV light and by spraying with reagents (Table 3). *p*-Coumaric, caffeic, and chlorogenic acids were confirmed by co-chromatography with authentic specimens.

Table 3. Phenolic acids of fermented cocoa.[a]

Acid	$R_f \times 100$						Color with chromatogenic reagent					
	HA[b]	EtAc[c]	BuAc[d]	HA[e]	BzAc[f]	Forestal[g]	UV	UV + NH_3	*p*-Nitro-aniline	Hopfner	AlCl$_3$	Benzidene
p-coumaric	72/45(73/40[6])	76(77[1])	89(88[1])	53(49[1])	—(62[2])	81(84[3])	Fv	Bl	B/Or	Y/Or	Bl	Or
Caffeic	63/30(63/30[1])	21(21[1])	78(78[1])	45(38[1])	32(29[2])	—(78[3])	Bl	Bl/Gr	Or/B	Or/Y	Y/Gr	Brick R
Chlorogenic	72/56(72/56[3])	0(0[1])	59(61[1])	65(64[1])	13(12[2])	—(—)	Gr/Bl	DEG	Or/B	Y/Or	Y/Gr	Y/B

[a] Present study (literature figures).
[b] 2% aqueous acetic acid.
[c] Ethyl acetate saturated with water.
[d] Butanol-acetic acid-water, 60:15:25 v/v.
[e] 10% aqueous acetic acid.
[f] Benzene-acetic acid-water, 60:70:30 v/v.
[g] Acetic acid-HCl-water, 30:3:10 v/v.

F = Faint
V = Violet
Bl = Blue
B = Brown
Or = Orange
Y = Yellow
Gr = Green
R = Red
DEG = Duck-egg Green

[1] Swain, 1953.
[2] Walker, 1962.
[3] Bate-Smith, 1962.
[4] Williams, 1955.
[5] Roberts, 1956.
[6] Smith, 1960.

DISCUSSION AND CONCLUSIONS

Previous investigations (Rohan, 1963) have shown that the aqueous methanolic soluble fraction of fermented cocoa beans

gives chocolate aroma when heated. The quality of the aroma was not affected by ethyl acetate extraction of the concentrate prior to heating, but there is now some evidence, from gas chromatographic studies, of quantitative effects. The significance of the results described must await assessment until the application of gas chromatography to evaluation of the various precursor groups is complete. A concurrent study of the flavonoids and phenolic acids of unfermented cocoa gave results identical with those described above for fermented cocoa except for the chloroform-insoluble (flavylogen) fraction. The unfermented sample contained a much higher proportion of the mobile (less complex) systems, which is in agreement with results of Forsyth (1955). The failure of unfermented cocoa to produce chocolate aroma when heated could invest this difference in flavylogen composition with some significance, but this too must await completion of the gas chromatographic studies before even tentative hypotheses can be advanced.

ACKNOWLEDGMENT

Specimens of quercetin, caffeic acid, chlorogenic acid, and *p*-coumaric acid were kindly supplied by Dr. T. Swain.

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Some Aromatic Compounds in Sap Composition of Maple Sap and Sirup

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(Manuscript received September 1, 1963)

SUMMARY

The constituents of a chloroform extract of maple sap were resolved by gas chromatography. The high-boiling components were found to be the aromatic compounds coumarin, vanillin, syringaldehyde, coniferyl aldehyde, and 2,6-dimethoxybenzoquinone. None were present in the sap in concentrations greater than 1 ppm. An ether-insoluble lignin was indicated by both chemical tests and infrared data. No significant differences were observed between the chromatograms of early and late sap extracts.

INTRODUCTION

In earlier publications the authors reported the presence of vanillin, syringaldehyde, and dihydroconiferyl alcohol in maple sirup. This initiated an investigation of the maple sap from which the sirup is made to find possible precursors of these compounds or other sap constituents that could be precursors of maple flavor. Skazin (1930) reported that the characteristic flavor of maple sirup and sugar is not found in the sap, but is developed during the atmospheric boiling process used to concentrate the sap to sirup. Experimental work in the authors' laboratory has substantiated this. Findlay and Snell (1935) had confirmed this, but could find no definite flavor precursor in the sap. Risi and Labrie (1935) also failed to find maple precursors in the sap during a series of studies on the production of maple sirup and sugar. The present paper reports the isolation and identification of aromatic compounds in sap of the maple tree that could be related to flavor components in the sirup.

EXPERIMENTAL

Extraction of the sap. As in the work on the flavor components of maple sirup, chloroform was used to obtain from sap an extract free of sugars and color. The trace amounts of compounds to be isolated necessitated that a large quantity of sap be extracted. Also, to minimize the chemical changes induced in it by microorganisms the sap had to be extracted immediately after exuding from the tree. Consequently, a special apparatus for extracting a large volume of sap was designed and installed in a sugar bush during the maple season. Two thousand gallons of sap from 300 trees was collected and transported by a system of plastic tubing to a tank and then into the extractor. The tank provided the needed constant pressure to cause the sap to flow through the extractor at a uniform rate. The extractor consisted of a galvanized steel cylinder 36 inches high and 10 inches in diameter, with a funnel-shaped bottom. The cylinder was half-filled with porcelain "saddles," and five gallons of ACS grade of chloroform was added. Sap from the holding tank entered the bottom of the extractor and bubled up through the chloroform and out a spout at the top. Since $CHCl_3$ is very slightly soluble in water, the sap in passing through the solvent gradually reduced its volume. Therefore, when the five-gallon batch of $CHCl_3$ in the extractor was reduced to 2.5 gallons, the solvent was removed and a new five-gallon lot of $CHCl_3$ added. It was possible to extract 500 gallons of sap per five-gallon charge of $CHCl_3$. Four such 2.5-gallon lots of extract were obtained during the 1962 maple season, representing early-, mid-, and late-season sap. These four lots of $CHCl_3$ were then brought to the laboratory and concentrated to 25 ml each by allowing them to stand at room temperature in a draft of air in a hood. The four lots were then held in glass-stoppered bottles for chemical and gas chromatographis examination.

Gas-liquid chromatographic (GLC) separations. The equipment and conditions for the gas chromatographic fractionation of the $CHCl_3$ extract of

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Table 1. Gas chromatographic operating conditions.

Model	Aerograph A-350 (with dual columns and thermal conductivity detectors)
Columns	Stainless steel, ¼″ O.D., 4′ long
Packing	20% Carbowax 20M on 60/80 mesh acid washed Chromosorb W
Temperatures	Injector—270°C Columns—90–240°C Detector—280°C
Flow Rate	100 ml He/min

maple sirup described by Underwood and Filipic (1963) were found satisfactory for the sap extract and are listed in Table 1. The Aerograph A350, using columns packed with either Carbowax 20M or SE-30, gave good separations, the Carbowax being the better of the two. A typical chromatogram resulting from the injection of 500 μl of the concentrated sap extract is shown in Fig. 1. The column was initially maintained 10 min at 90° after injection of sample to allow complete elution of the very large solvent peak, and the temperature was then programmed at the rate of 4° per min to 240°C, at which setting it was held until the last peak was obtained (about 65 min).

Since there was a possibility that some of the peaks obtained were due to the high injection temperature, the following experiment was performed. A 1-ml portion of the concentrated extract was sealed in a thick-walled glass tube under nitrogen. The tube was inserted into a Carius apparatus, brought to 300°C in about 4 hr and then allowed to cool to room temperature. A chromatogram obtained using 500 μl of this heated extract was compared to that from the unheated sample. Fractions whose peak heights increased significantly were considered possible artifacts resulting from the high injection temperature. These peaks are labeled "A" in Fig. 1.

RESULTS AND DISCUSSION

GLC isolates. Attention was focused on the higher-boiling compounds (peaks 7–13) since they were easier to isolate for physical identification. As shown in Table 2, diethyl phthalate, coumarin, vanillin, dibutyl phthalate, syringaldehyde, coniferyl aldehyde, dioctyl phthalate, and 2,6-dimethoxybenzoquinone were present in this extract of maple sap. These identifications, based on infrared spectra, were confirmed by cochromatography of the individual fraction with the indicated compound on a polar (Carbowax 20M) and comparison of retention times on a nonpolar (SE-52) column. Concentrations of these compounds were estimated to be less than 1 ppm, based on comparison of peak areas with standards of known concentration. In the case of vanillin, the detection of the characteristic odor plus the fact that its presence in sirup had been established, strengthened its identification. The coumarin fraction also exhibited its characteristic bitter taste and sweet odor, while the quinone fraction consisted of orange crystals of metallic luster which dissolved to give a strong yellow color in chloroform. The vanillin and coumarin

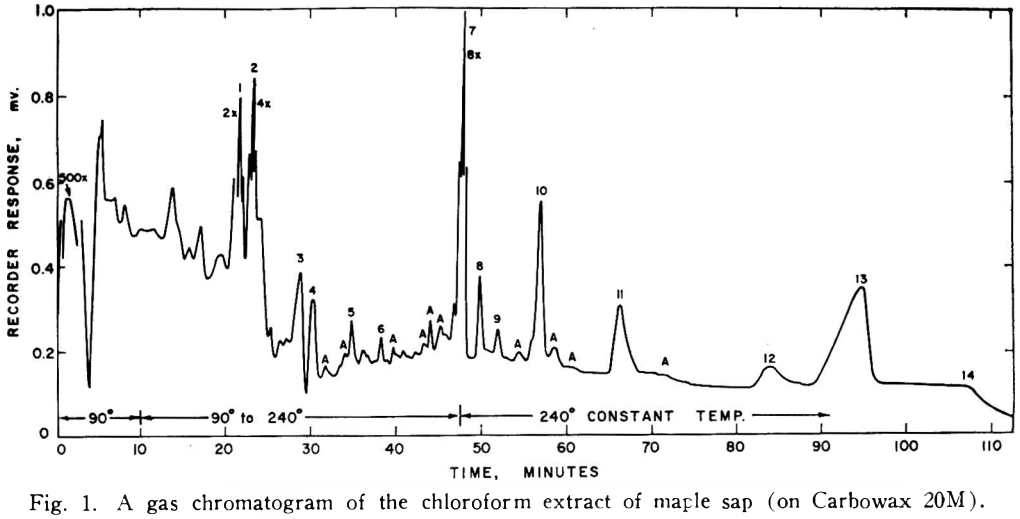

Fig. 1. A gas chromatogram of the chloroform extract of maple sap (on Carbowax 20M).

Table 2. Identification of maple sap components.

Peak no.	Identity	Evidence		
7	Diethyl phthalate	R.T.,[a]	Co.,[b]	I.R.[c]
8	Coumarin	R.T.,	Co.,	I.R., Odor[d]
9	Vanillin	R.T.,	Co.,	I.R., Odor
10	Dibutyl phthalate	R.T.,	Co.,	I.R.
11	Syringaldehyde	R.T.,	Co.,	I.R.
12	Coniferyl aldehyde	R.T.,	Co.,	I.R.
13	Dioctyl phthalate	R.T.,	Co.,	I.R.
14	2,6-Dimethoxybenzoquinone	I.R.,	Color[e]	

[a] Retention time of the collected fraction equal to that of a known on an SE-52 column.
[b] Cochromatographed on a Carbowax 20M column.
[c] Comparison of spectra with authentic samples.
[d] Characteristic odor detected.
[e] Intense color of solid and chloroform solution noted.

found in the sap could contribute to the flavor of the sirup. Of the other GLC isolates, coniferyl aldehyde oxidizes readily to vanillin, and so can be considered a possible flavor precursor.

Peaks 7, 10, 13 (Fig. 1) have been respectively identified as diethyl, dibutyl, and dioctyl phthalate esters. Such compounds are commonly used plasticizers and may have been "dissolved" by the sap while in the plastic tubing collection lines. To determine whether this could be the case a $CHCl_3$ extract was obtained from sap that had not been in contact with plastic tubings that might contain phthalate esters. No dioctyl phthalate was noted in this extract. However, two fractions did cochromatograph with added diethyl and dibutyl phthalate, but not enough material was available for infrared absorption data. In spite of this evidence to the contrary the authors feel that these two phthalates are also contaminants of unknown origin.

All fractions from the Carbowax column collected above 200°C were contaminated by substrate bleed. This contamination was so great that it was not possible to obtain good infrared curves. Therefore, these fractions were individually redissolved in $CHCl_3$ and rechromatographed on an SE-52 column. Pure fractions resulted, which gave much improved infrared curves.

The amounts collected, however, were in the submilligram range and required special techniques for infrared analysis. The fractions identified as coumarin and coniferyl aldehyde were run in carbon disulfide solution in ultramicro "D" cells (0.05 mm path length) (Connecticut Instrument Company, Wilton, Connecticut). Chloroform solutions of the vanillin and 2,6-dimethoxybenzoquinone fractions were evaporated to dryness in the cavity of the cell. The location of the infrared bands for the latter fraction agreed with those reported by Flaig and Salfeld (1959). Also, the quinone prepared from 2,6-dimethoxyphenol by the method of Davidge *et al.* (1958) gave an identical spectrum. Syringaldehyde was present in the greatest concentration, and the amount isolated was sufficient to coat (by evaporation from chloroform solution) a specially designed micro salt window of greater area than the "D" cell. The detailed spectrum obtained was completely identical with that of a standard. In each case, the infrared spectrum of the fraction was compared with that of a known compound treated in the same manner. Work is being pursued on identification of the constituents of lower retention time, representing the more volatile compounds in the extract.

Lignin component. The $CHCl_3$ extracts of sap gave the classical Weisner phloroglucinol-HCl reaction for lignin, but lignin was not detected in any fraction separated by GLC. Therefore, an effort was made to isolate this Weisner-positive component by chemical means. The addition of four volumes of diethyl ether to the $CHCl_3$ extract precipitated a fluffy white material. This was isolated by filtration through sintered glass, but upon drying it turned a light brown and was only partially resoluble in $CHCl_3$. The material precipitated by ether

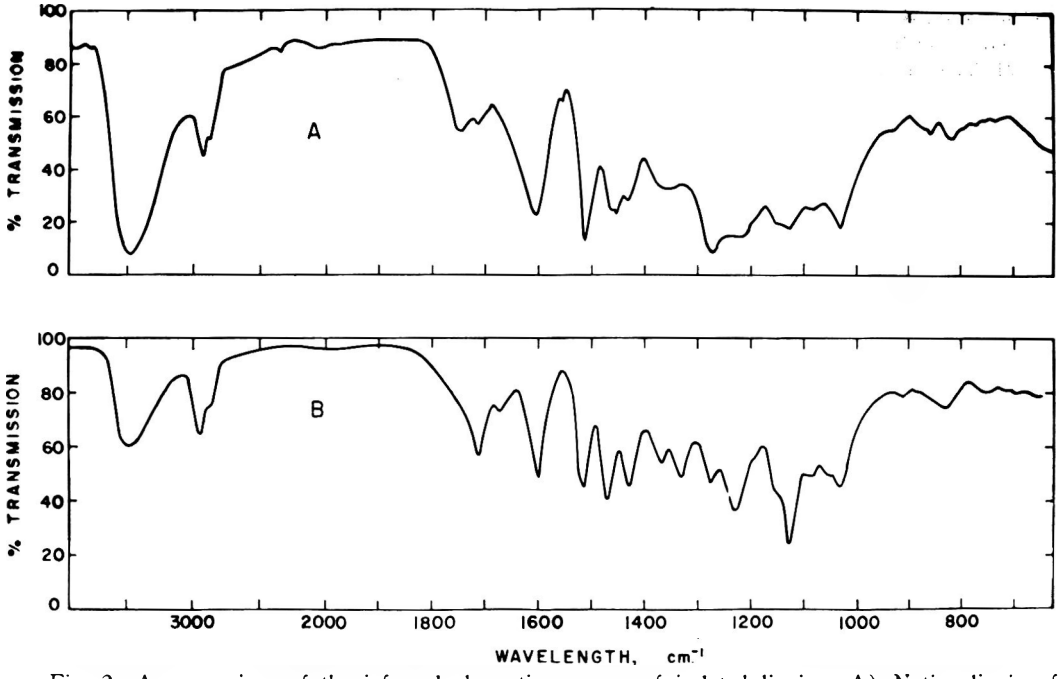

Fig. 2. A comparison of the infrared absorption curves of isolated lignins. A) Native lignin of western hemlock isolated by Hergert; B) Lignin from maple sap.

was therefore separated by centrifugation. The precipitate, when redissolved in $CHCl_3$, gave a strong phloroglucinol-HCl reaction whereas the ether-chloroform soluble fraction gave only a faintly positive reaction. This positive reaction of the ether-chloroform solubles could be due to either a small amount of lignin not removed by this procedure or to the minute quantity of coniferyl aldehyde present in the solution. The infrared spectrum of this precipitate was similar in many respects to those of lignin preparations by Kudzin *et al.* (1951) and by Kolboe and Ellefsen (1962).

The spectrum obtained for native lignin by Hergert (1961) is compared with that obtained by the authors (Fig. 2). Findlay and Snell (1935) reported the possible presence of such material in maple sap based on chemical color reactions alone. This more specific infrared evidence has confirmed their finding. Vanillin, syringaldehyde, and dihydroconiferyl alcohol are well known degradation products of lignin. The relationship of the lignin component as the possible precursor to these and other aromatic compounds in sap and sirup and to the as yet unidentified maple flavor constituents of sirup, will be pursued in further studies.

ACKNOWLEDGMENTS

The authors thank Dr. John C. Pew, Forest Products Laboratory, for providing a sample of coniferyl aldehyde, and Jesse E. Ard for obtaining the infrared absorption curves of syringaldehyde and lignin.

Reference to company product names does not imply aproval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

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Effect of Temperature on Radiosensitivity of Salmonella Typhimurium [a]

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(Manuscript received August 16, 1963)

SUMMARY

Radiosensitivity of *S. typhimurium* increased as a function of irradiation temperature from 32 to 130°F. The greatest effect of temperature during irradiation occurred at temperatures above 110°F. Rate of bacterial destruction was significantly greater when the ionizing energy and thermal energy were applied simultaneously than when they were applied consecutively.

INTRODUCTION

In an effort to lower the irradiation sterilization requirement for foods, various attempts have been made to sensitize bacteria to radiation by coupling the irradiation treatment with some other lethal treatment such as exposure to antibiotics, ultrasonic energy, etc. Much data reported in the literature demonstrate the effectiveness of ionizing energy followed by thermal energy (Morgan and Reed, 1954; Kempe, 1955; Kan *et al.*, 1957).

Some studies have been reported on the effect of temperature during irradiation on the destruction of microorganisms; however, the investigations were usually carried out over a span ranging from sub-zero temperatures to about 104°F. Not much has been reported with regard to the simultaneous application of ionizing energy and thermal energy at temperatures at which thermal destruction of bacteria is rapid.

Adams and Pollard (1952) observed that the radiation sensitivity of a bacteriophage increased sharply at irradiation temperatures above 113°F. The radiation sensitivity of a yeast, *Saccharomyces cerevisiae*, was also found by Wood (1954) to increase at irradiation temperatures in excess of 113°F. Setlow (1952) irradiated catalase at various temperatures in the dry state, and his results indicated a sharp rise in radiosensitivity at about 113°F and another rise at about 212°F. He attributed the increase in radiosensitivity at these two regions of temperature to correspond respectively to inactivation of half and all the enzyme activity of the molecule.

With regard to consecutive application of ionizing energy and thermal energy, Giese and Crossman (1946) suggested the following mechanism: The irradiation treatment causes the rupture of chemical bonds in some vital protein molecule. The heat causes increased agitation of the molecule, and it disintegrates. From this viewpoint it would seem more efficient to apply the thermal and ionizing energy simultaneously, because in a state of rapid oscillation a protein molecule would probably be more vulnerable to attack by other destructive agents and also there would be no repair of radiation damage such as might occur when irradiation is carried out at ambient temperatures.

Therefore, this investigation was made to determine the radiosensitivity of bacterial cells at elevated temperatures and also to compare the lethal effect of simultaneous irradiation and heating with the process whereby irradiation and heat are applied in succession. A *Salmonella* species was chosen as the test organism on the basis that if simultaneous irradiation and heating did prove to be an effective method for inactivating bacterial cells, a potential application of this technique might be in rendering *Salmonella* non-viable in liquid egg prior to freezing or drying.

[a] This research was supported by N.I.H. Grant No. EF00006-05. Contribution number 557 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts.

PROCEDURE

Test organism. The organism used was *Salmonella typhimurium* ATCC 6994. The cells were grown in Roux flasks on trypticase soy agar supplemented with yeast extract at a concentration of 10 g/L. After a four-day incubation period at 98–99°F, the cells were washed off the surface of the agar with phosphate buffer pH 7.0, washed in phosphate buffer, and finally resuspended in phosphate buffer. The stock cell suspension was maintained at 36–40°F in a screw-cap bottle containing glass beads. There was no appreciable loss in viable count during the one-month period the cells were used.

Sample preparation. Two different substrates were used, whole egg magma and liquid yolk, both prepared from freshly broken out eggs. The pH ranged from 7.6 to 7.8 for the liquid whole egg, and from 6.15 to 6.25 for the yolk.

Twenty-five hundredths ml of the inoculated substrate was filled with a micrometric syringe into thin-walled melting-point capillary tubes, and the tubes were sealed in the flame of a micro burner. The inoculum per tube was of the order of 25 million cells.

Irradiation of samples. The source of irradiation was a Mark I cobalt-60 food irradiator furnished by the Atomic Energy Commission, and the dose rate was approximately 5000 rads per minute. The irradiation container had an insulated jacket of glass wool, and, in addition, a styrofoam block (6 × 14 × 14 inches) was made to fill the void inside the steel container. A cylinder had been cut out along the central axis of the styrofoam block so as to accommodate a half-pint vacuum jar.

For irradiation at different temperatures the vacuum jar was filled with water adjusted to the desired temperature. Five sample capillary tubes were then placed in the vacuum jar, and the samples were irradiated for a prescribed length of time. Temperature during irradiation was measured with a thermocouple. There was virtually no heat loss in the system during the maximum holding time at 110°F or less. At the highest heating temperature employed, 130°F, there was about a 1.5°F temperature drop during the maximum holding time of 18 min. At intermediate temperatures and holding times, the temperature fluctuation was proportionately smaller. Upon completion of the irradiation the capillary tubes were plunged immediately into ice water and held until bacterial counts were made.

In the experiments in which irradiation and heating were carried out separately, the tubes were first irradiated in the vacuum jar containing ice water (32°F) and then heated for the same length of time that was required to deliver the irradiation in the insulated vacuum jar containing water at some specified temperature. After heat treatment, the tubes were immediately transferred to an ice water bath.

Bacterial counts. After irradiation and heating, the capillary tubes were submerged in chromic acid cleaning solution for several minutes, rinsed in running tap water, and then transferred to a culture tube containing 10 ml of chilled sterile phosphate buffer, pH 7.0. Each capillary tube was crushed with a glass rod, and the culture tube was shaken vigorously 50 times. Serial dilutions were made using dilution blanks of chilled phosphate buffer pH 7.0, and, from the appropriate dilutions, one-tenth-ml portions were spread over the surface of pre-poured plates with bent glass rods. Four replicate capillary tubes were plated out for each irradiation-heating treatment. Trypticase soy agar supplemented with yeast extract (10 g/L) was the culture medium, and plates were incubated at 98–99°F for 48–72 hr before the colonies were counted.

RESULTS AND DISCUSSION

Fig. 1 shows the survival curves obtained when cells of *S. typhimurium* suspended in egg yolk were irradiated at various temperatures ranging from 32 to 130°F. The curves appear to be convex, a type of survival curve that has usually been explained on the basis of a population consisting of two groups of different resistances. However, Setlow (1952) irradiated catalase at different temperatures, and some of the survival curves he obtained were convex. Since catalase is a molecule and not a living entity, it becomes difficult to explain the convex shape of the survival curves on the basis of a population of mixed resistance, because one would imagine a general uniformity to exist among similar molecules except with regard to activation energy. Nevertheless, a regression line was drawn by the method of least squares through all of the points except the control point, and this is what is represented in Fig. 1. Each of the points is the average count of four replicate capillary tubes.

Fig. 2 shows the survival curves that resulted when cells of *S. typhimurium* were irradiated at various dose levels in egg yolk at 32°F and then heated at different temperatures for the same length of time that was required to deliver the irradiation. Here again, as in Fig. 1, the survival curves

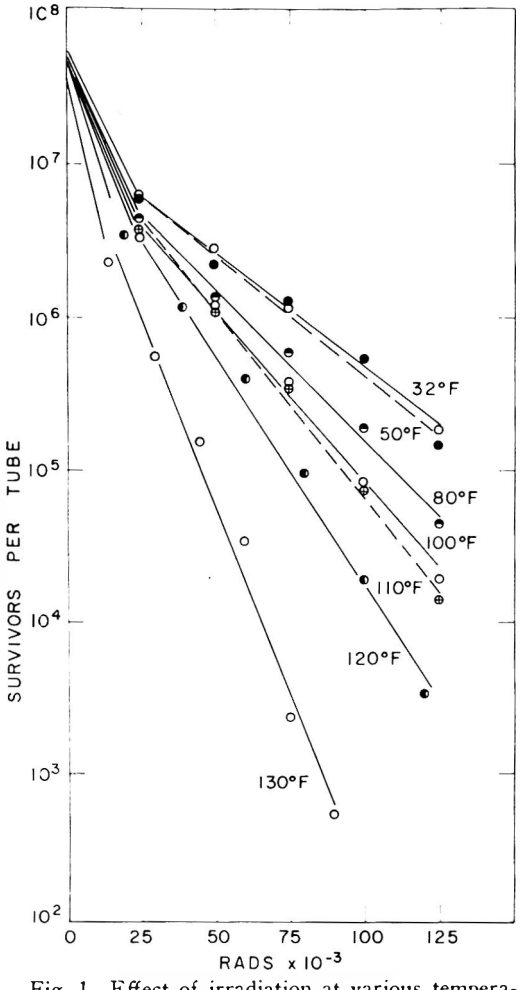

Fig. 1. Effect of irradiation at various temperatures on survival of *S. typhimurium* in egg yolk.

appeared to be convex, and the regression line was constructed on all survival points except the control point.

For similar heating temperatures the survival curves plotted in Figs. 1 and 2 can be compared to determine which treatment is the more destructive, because in both cases the same amounts of ionizing energy and thermal energy had been administered, the only difference being in the order of delivery. Since each survival curve consisted of two different slopes, the linear regression slopes determined on the second part of the survival curves were compared by means of a *t*-test. A significant difference at the 1% level was found for the two different treatments carried out at either 100 or 130°F. The different treatments at intermediate temperatures were not tested, but inspection indicated that they too would be significantly different.

The survival rate during irradiation at lethal temperatures is the integral sum of three different components, that is, the effect of irradiation, the effect of heat, and the effect of an interaction between irradiation and heat. On the assumption that these effects are additive, determining the survival rate with heating alone at a given temperature and subtracting this from the over-all survival rate would remove the component effect of heat. The extent of the interaction between heat and irradiation can then be determined by plotting the survival rates corrected for the heating effect as a function of irradiation temperature. This has been done in Fig. 3 except that decimal reduction dose, which has been defined as the radiation dose required to destroy 90% of the population, and which is also the negative reciprocal of the slope of the survival curve,

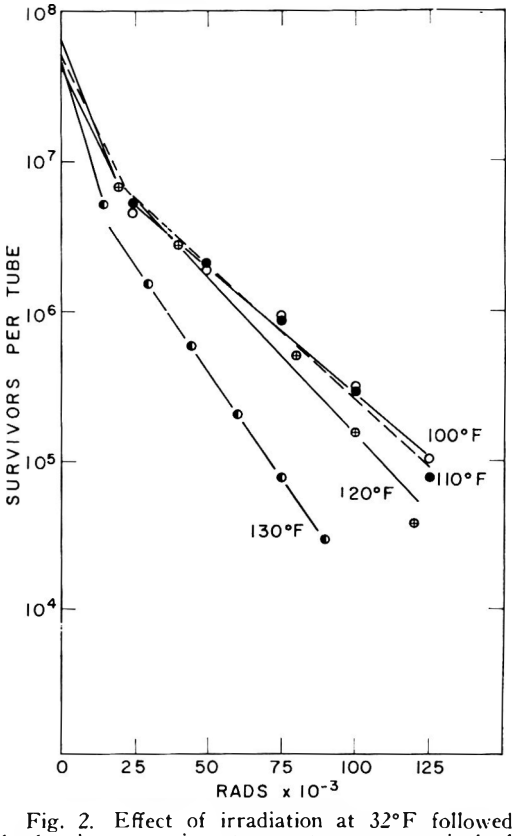

Fig. 2. Effect of irradiation at 32°F followed by heating at various temperatures on survival of *S. typhimurium* in egg yolk.

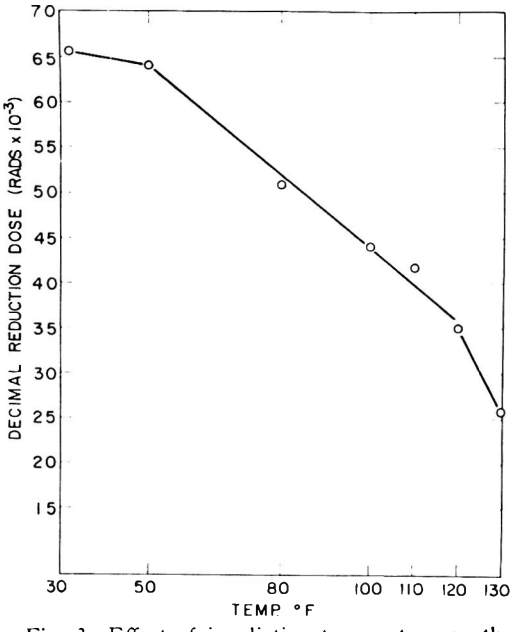

Fig. 3. Effect of irradiation temperature on the decimal reduction dose of *S. typhimurium* in egg yolk.

has been plotted as a function of irradiation temperature. Radiosensitivity of *S. typhimurium* seemed to increase in a nonlinear fashion as irradiation temperature increased over the span of 32 to 130°F. However, the effect was greatest when irradiation was carried out at temperatures above 120°F. Adams and Pollard (1952) also observed a sharp rise in radiosensitivity of a bacteriophage when irradiating at temperatures beyond 120°F. By plotting the inverse slopes of the initial portion of the irradiation survival curves as a function of temperature, a similarly shaped curve resulted except that it was displaced lower on the graph. This curve is not shown.

Fig. 4 shows the survival curves for *S. typhimurium* irradiated at various temperatures in whole-egg substrate. Fig. 5 presents the survival curves obtained in whole egg when irradiation and heating were applied separately. The shape of the survival curves is similar to that obtained in egg yolk, yet the cells used in the whole-egg study were from a different harvest. Each survival point represents the average of four capillary tubes, and regression lines were drawn through all points except the control point. A comparison of regression slopes by a *t*-test indicated that destruction was significantly greater (1% level) when irradiation was carried out at 130°F than when the cells were irradiated at 32°F and then heated for an equivalent time at 130°F. Results were similar for survival rates at 100°F. Survival rates at intermediate temperatures were not compared statistically.

The effect of irradiation temperature on the decimal reduction dose of *S. typhimurium* in whole egg is shown in Fig. 6. This curve was constructed in the same manner as that in Fig. 3, that is, irradiation-heating sur-

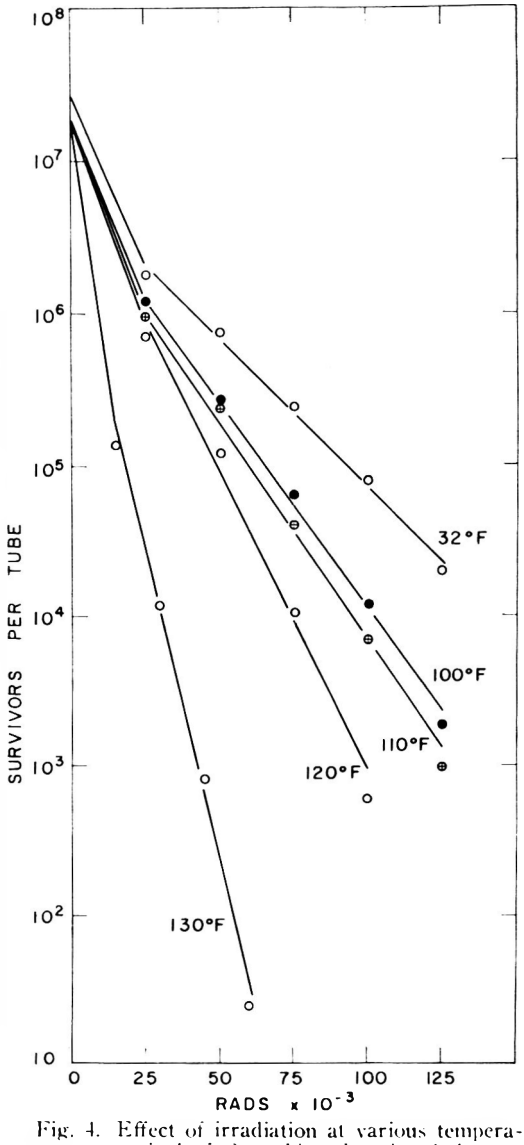

Fig. 4. Effect of irradiation at various temperatures on survival of *S. typhimurium* in whole egg.

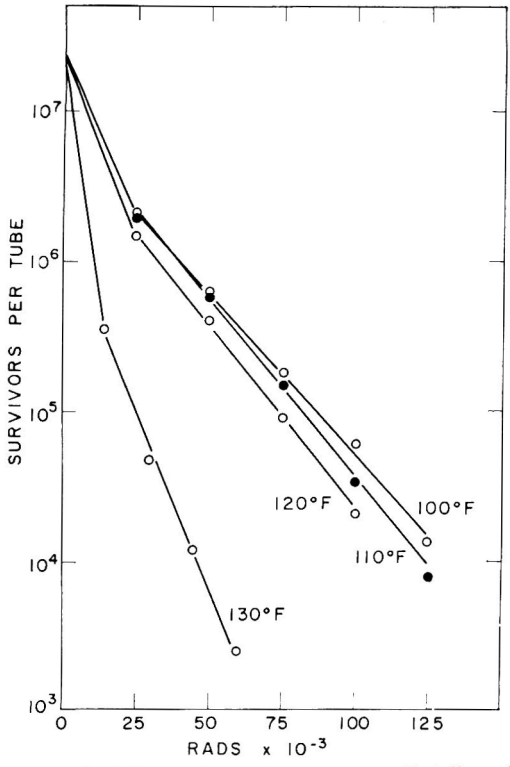

Fig. 5. Effect of irradiation at 32°F followed by heating at various temperatures on survival of *S. typhimurium* in whole egg.

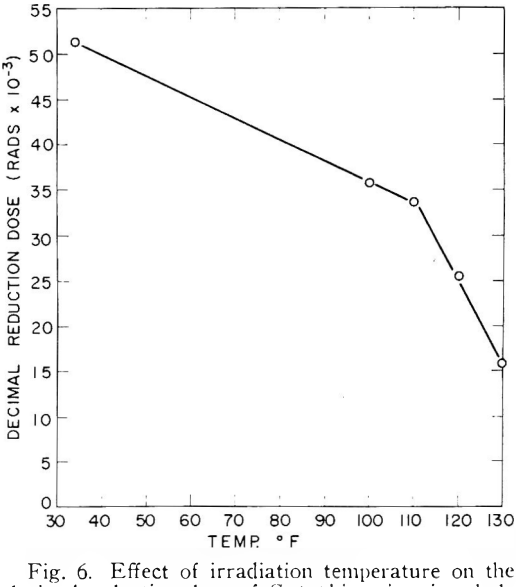

Fig. 6. Effect of irradiation temperature on the decimal reduction dose of *S. typhimurium* in whole egg.

vival rates were corrected for temperature effect and then converted to decimal reduction dose. The radiosensitivity in whole egg increased as the irradiation temperature increased but a sharp rise in radiosensitivity occurred at a temperature of 110°F or greater. This is interesting because in this temperature region the thermal inactivation of *S. typhimurium* proceeded at a noticeable rate. In whole egg, for example, after 25 min of exposure to 100, 110, 120 or 130°F, the percent survival of *S. typhimurium* was 100, 80, 30, and 0.2. Thus, the most significant effect of temperature during irradiation occurred when irradiation was carried out at a temperature in the lethal range for *S. typhimurium*. The shape of the curve for whole egg (Fig. 6) is generally similar to the curve for egg yolk (Fig. 3). However, the curve for egg yolk is displaced above the curve for whole egg, indicating that the organism is more resistant in egg yolk. The increased resistance in egg yolk is probably dependent on the low pH of the egg yolk.

Various theories have been proposed to explain the increased radiosensitivity at high temperatures, including: a) increased diffusion of free radicals; b) excitation, which is not considered important at low temperatures, becomes of importance at high temperatures; c) increase in target size due to thermal expansion; d) formation of new radical species; and e) increase in bond length at high temperature makes molecule unstable. Each of these factors may play a minor role in contributing to the total effect. For example, it has been shown by calculation that there can be a cubical expansion of biological matter with an increase in temperature; however, the theoretical increase in volume of the "target" is not sufficient to account for the observed increased radiosensitivity.

Adams and Pollard (1952) proposed a model that merits serious consideration. They stated that denaturation of protein by heat requires the rupturing of three adjacent residue bonds in the molecule. This causes the main chains to drift apart, and the molecule loses its biological configuration. If one or more bonds are broken by ionizing energy, then the requirement for thermal energy is reduced.

In the case where irradiation is administered prior to heating, there can be a repair

of bonds broken by ionizing energy, and this could explain why simultaneous irradiation-heating is the more efficient process in terms of bacterial destruction.

One of the major problems involved in sterilizing foods by radiation is the incomplete destruction of proteolytic enzymes with a sterilizing dose of four to five million rads. Since proteolytic enzymes are essentially proteins, it would be of interest to determine if they could be inactivated by irradiating at moderate temperatures.

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Some Aspects of the Action of Tylosin on Clostridium Species PA 3679

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(Manuscript received May 27, 1963)

SUMMARY

Spores of PA 3679 were derived from vegetative cells made resistant to an initial 4.0 ppm tylosin. Rate of germination of PA 3679 spores, either from the original sensitive stock or from a resistant stock, is not affected by tylosin. Vegetative cells of PA 3679 arising from germination and subsequent outgrowth of the resistant cells are killed by 0.8–0.9 ppm of residual tylosin. This is to be compared with 0.05–0.25 ppm initial tylosin required to kill sensitive cells. The residual level of tylosin in sterile spore-recovery media is dependent on the initial tylosin concentration and the medium.

INTRODUCTION

Tylosin is a new antimicrobial agent that has been described previously (Hamill *et al.*, 1961; McGuire *et al.*, 1961). An earlier observation by Malin (1958) of the destruction of sporeforming bacteria by tylosin has been confirmed by Denny *et al.* (1961b) and by Greenberg and Silliker (1962). There is disagreement on the mode of action of tylosin. Denny *et al.* indicated that their results with tylosin may be interpreted on the basis of an acceleration in the rate of spore destruction by heat or, alternately, an inhibition of spore germination in the heat-treated growth medium. Subsequently, Denny *et al.* (1961a) demonstrated that tylosin had no effect on spore destruction rates. In contrast, Greenberg and Silliker reported that tylosin has no effect against spores, *per se*, but attacks cells at the stage of their life cycle when they are capable of outgrowth. This report presents evidence supporting the sypothesis that the rate of spore germination is not affected by tylosin in an environment where germination can take place. Also considered in this study is the effect of tylosin concentrations on vegetative cells arising from spore germination and subsequent outgrowth.

MATERIALS AND METHODS

Tylosin was used as the water-soluble lactate salt. Solutions were sterilized by passage through bacteriological fritted glass filters before use. Aliquots of the solutions were added to previously sterilized brain heart infusion broth (Difco) to give the desired concentration of tylosin.

Clostridium species National Canners Association putrefactive anaerobe 3679 (PA 3697) was used in the study. Spore stocks were prepared according to the method of Zoha and Sadoff (1958), washed twice in sterile deionized water, and stored in the refrigerator.

The reaction vessels were screw-cap test tubes containing 25 ml of fluid thioglycollate medium (Difco) when filled, or 20 ml brain heart infusion broth overlaid with sterile "vaspar." After inoculation, each tube was heat-shocked 15 min at 185°F. Samples were removed periodically and dilutions made in sterile deionized water. In experiments where both spore and total viable cell counts were determined, a single sample was withdrawn and diluted in sterile deionized water for subsequent handling. Spores were defined as those organisms capable of surviving 15 min at 185°F. Pasteurization of the dilution tubes, from which the corresponding total count samples were taken, was done by placing in a 185°F water bath.

PA 3679 was quantified by 5-tube most-probable-number determinations (Am. Publ. Health Assoc., 1955) in fluid thioglycollate medium (Difco) incubated 7 days at 98.6°F. Development of both turbidity and putrefaction constituted a positive result.

Tylosin concentrations were measured by a turbidimetric assay procedure with *Staphylococcus aureus* H (ATCC 9144) as the test organism (McGuire *et al.*, 1961). This is a rapid assay (4 hr at 98.6°F) with a sensitivity extending to 0.6 ppm tylosin.

RESULTS AND DISCUSSION

Preliminary results obtained with PA 3679 are shown in Fig. 1. PA 3679 spores

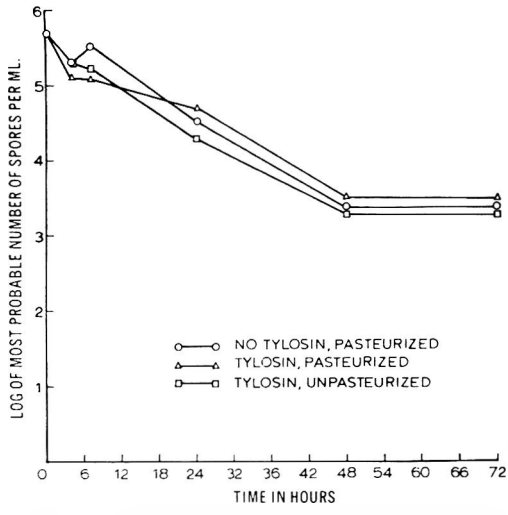

Fig. 1. Effect of tylosin on rate of germination of spores of PA 3679 in brain heart infusion broth. Initial conc. of tylosin 10 ppm.

were suspended in brain heart infusion broth containing 0 and 10 ppm tylosin. There was no acceleration or inhibition of the normal spore germination rate, as measured by surviving spores. All of the tubes in the tylosin series were negative. No growth was observed in the tubes of the tylosin series, thus no viable cells resulted from the spores which started the germination process in the presence of the antibiotic. Since there was no vegetative growth in the tylosin series, the data demonstrate that PA 3679 cells were particularly sensitive to tylosin before exponential outgrowth occurred, and the data are in agreement with those of Greenberg and Silliker (1962).

The next phase was to determine if PA 3679 vegetative cells could become resistant to tylosin, and if so, observe whether spores derived from vegetative cells resistant to tylosin would show outgrowth, and if the resulting vegetative cells would also be resistant to tylosin.

Positive growth was observed when tubes of brain heart infusion broth containing 0.05 ppm tylosin were inoculated with PA 3679 spores. Subsequent transfers of these vegetative cells to increasingly higher levels finally resulted in cells of PA 3679 which would grow in the presence of an initial 4.0 ppm tylosin. Growth at levels higher than this could not be attained despite numerous attempts. A considerable time lag was also observed before the onset of growth in increasingly higher levels of tylosin.

PA 3679 cells which grew in the presence of an initial 4.0 ppm tylosin were transferred several times in brain heart infusion broth to ensure these were not tylosin-dependent. Spores prepared from these PA 3679 cells were used to inoculate a series of brain heart infusion broth tubes containing 0, 0.05, 0.25, 0.5, 2.5, and 4.0 ppm tylosin. A similar series was also prepared and inoculated with PA 3679 spores of the original sensitive stock suspension. Positive growth was observed in all of the tubes in the former series, whereas in the latter series only the first tube (0.05 ppm tylosin) showed positive growth.

In the first phase of the study of development of resistance to tylosin, an unexpected event was noted: the time lag observed before the onset of growth when transfers were made from a lower to a higher concentration of tylosin. If tylosin was not destroying PA 3679 vegetative cells, perhaps these cells were inhibited only at a particular concentration of tylosin. Conceivably, the reason for growth following a time lag could be due to a decrease in concentration of the initial tylosin to a lower level where growth could occur.

Since the possible decrease in initial tylosin concentration might be dependent on the growth medium used, the residual concentration was periodically measured in several commonly used growth media. Sterile brain heart infusion broth, pork-pea infusion medium (Wheaton, 1959), fluid thioglycollate medium and trypticase soy broth (BBL) were used. Each series of tubes contained 0, 3.75, 7.5, 15, 30, or 45.6 ppm tylosin in 25 ml. The tubes were not inoculated. Table 1 shows the residual tylosin concentration for each series. Fig. 2 depicts the stability data for the initial 7.5-ppm tylosin series. These curves are typical of the stability study and show an initial rapid decrease in concentration to a level dependent on the initial concentration and the medium. Thereafter, there is a slowly falling level over a long period.

The concept that vegetative cells of PA 3679 would be inhibited by tylosin only if

Table 1. Stability of tylosin in sterile growth media.

Initial tylosin concentration	Medium[a]	Residual tylosin, ppm: Days 0	1	3	10	21	50
3.75 ppm	(1)	2.2	1.7	1.3	.9	.7	<.6
	(2)	2.6	2.5	1.9	1.4	1.2	<.6
	(3)	1.7	2.1	1.6	1.1	1.3	<.6
	(4)	2.6	2.2	1.3	1.0	.9	<.6
7.5 ppm	(1)	5.2	3.6	2.7	1.9	1.4	1.2
	(2)	6.7	5.6	4.4	3.1	2.3	1.6
	(3)	3.8	3.2	4.0	2.9	2.3	1.5
	(4)	5.8	4.3	3.2	2.3	1.8	1.5
15 ppm	(1)	11.0	11.0	6.0	3.3	3.0	2.6
	(2)	13.4	12.0	9.2	6.6	4.7	3.3
	(3)	8.2	8.4	5.8	3.5	5.2	3.5
	(4)	12.3	9.0	6.6	4.8	3.5	3.1
30 ppm	(1)	22.3	17.0	13.0	9.3	7.5	5.6
	(2)	29.0	28.3	20.0	14.3	11.0	7.9
	(3)	17.5	19.8	16.0	10.0	12.7	8.4
	(4)	26.5	18.5	16.0	9.6	7.7	6.7
45.6 ppm	(1)	33.3	24.6	19.0	12.0	11.4	8.9
	(2)	40.9	39.9	28.9	22.0	16.9	13.2
	(3)	31.3	26.3	20.6	17.2	17.6	12.2
	(4)	38.6	33.3	23.9	17.4	14.6	11.9

[a] 1 = brain heart infusion broth; 2 = pork-pea infusion broth; 3 = fluid thioglycollate; 4 = trypticase soy broth.

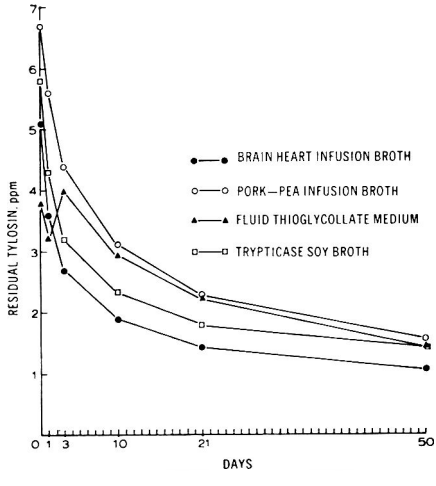

Fig. 2. Stability of tylosin. Initial conc. of tylosin 7.5 ppm.

the concentration remained above a minimum level was tested next. Outgrowth and subsequent vegetative cell growth of the original sensitive stock of PA 3679 spores was restricted by an initial level of 0.25 ppm tylosin, well below the sensitivity of the turbidimetric assay for tylosin. Use of the resistant stock of PA 3679 would therefore permit assay for residual tylosin if the residual level was not less than 0.6 ppm.

A series of tubes containing an initial concentration of 10 ppm tylosin in brain heart infusion broth (20 ml) was inoculated with 170,000 spores per ml from the resistant stock of PA 3679 spores. Pasteurized and unpasteurized tubes were assayed for most-probable-number determinations and residual tylosin. Fig. 3 illustrates the progression of spore germination and outgrowth. Fig. 4 shows the residual tylosin levels at each sampling period. After fifteen days, there were a small number of viable spores and 1.7 ppm residual tylosin still remaining. No positive growth had been observed in any of the tubes.

In an attempt to decrease the induction period, one ml from a 16-day tube was added to a series of brain heart infusion broth tubes (20 ml) containing initial concentrations of 0, 3, 4, and 5 ppm tylosin. Each tube contained approximately 65 spores per ml. Fig. 5 shows the decrease in tylosin concentration and the time of outgrowth and subsequent cell growth. At each time, growth occurred when the residual tylosin level was 0.75–

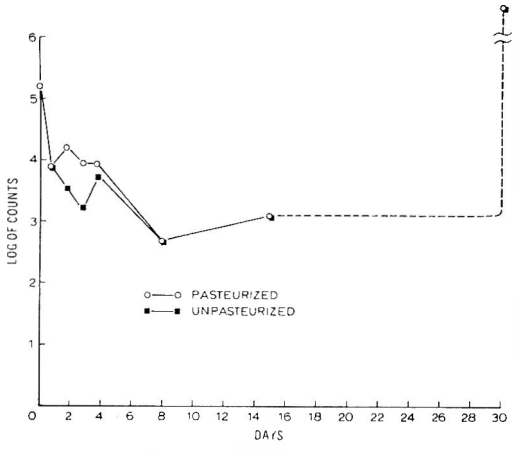

Fig. 3. Viability of spores of PA 3679 in brain heart infusion broth. (In the presence of tylosin, initial conc. 10 ppm.)

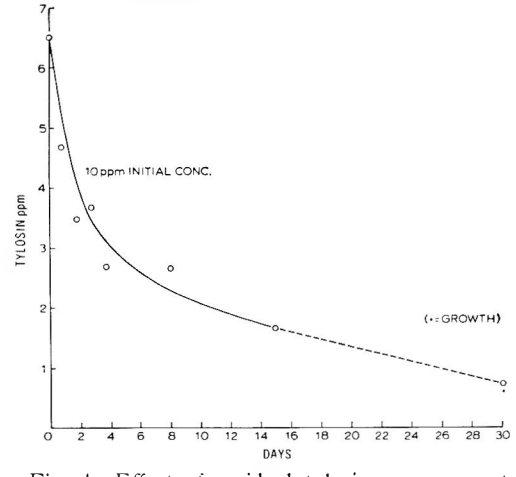

Fig. 4. Effect of residual tylosin conc. on outgrowth of spores of PA 3679 in brain heart infusion broth. (Initial conc. tylosin 10 ppm.)

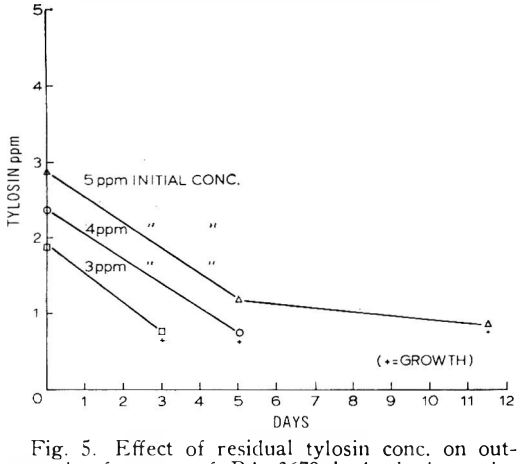

Fig. 5. Effect of residual tylosin conc. on outgrowth of spores of PA 3679 in brain heart infusion broth.

0.85 ppm. It is interesting to note that in the series shown in Figs. 3 and 4, growth occurred after approximately the same elapsed time (20–30 days) and at approximately the same residual tylosin level. (The dotted line is a projected stability curve based on data given in Table 2.)

It is evident that the stability of tylosin will vary from medium to medium. The rates of degradation, an early rapid decrease followed by a prolonged slow decrease, are similar regardless of the medium used. However, the final residual level attained is dependent on the initial concentration and the medium.

In addition to factors previously reported, i.e., pH, temperature, and indigenous population, it would appear that each food product will also be a factor in determining the specific level of tylosin to be used.

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Survival of Clostridium perfringens in Frozen Chicken Gravy[a]

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(Manuscript received November 5, 1963)

SUMMARY

The effect of freezing *Clostridium perfringens* in chicken gravy was examined. Two means were used in inoculating the gravy: 1) inoculum of *C. perfringens* was allowed to grow in the gravy for 6 hr; and 2) spores produced in Ellner's medium and dried on sterile soil were added to the gravy. In both cases, the gravy was then held in the frozen state (−17.7°C) for 180 days. When growth was permitted in the gravy, a maximum of 4.29% and 3.69% of viable cells survived for 90 and 180 days, respectively. When spores dried on soil were added to the gravy, maximum survival was 37.9% at 90 days and 10.9% at 180 days.

INTRODUCTION

The response of *Clostridium perfringens* to freezing temperatures is controversial. Raj and Liston (1961) reported little change in the viable count of *C. perfringens* frozen (−30°F) in fish homogenates and held at freezing temperatures (0°F). Kemp *et al.* (1962) stated that the freezing of bacteriological specimens may make the recovery of this organism extremely difficult. They suggested that epidemiologists investigating food poisoning outbreaks presumably due to *C. perfringens* should refrigerate food samples rather than freezing them. Canada *et al.* (1963) reported on freezing and holding experiments involving the vegetative cells and spores of four strains of *C. perfringens* in various suspending media. After a holding time of 48 hr, the maximum survival for vegetative cells in various suspending media was 2.4%. The survival rate for spores under similar conditions was 16–58%.

This study was undertaken to obtain more information on the effects of a single freezing and thawing procedure on a population of *Clostridium perfringens* which had been grown or held in a natural food medium.

EXPERIMENTAL METHODS

Series I. The food incriminated in poisoning incidents caused by *C. perfringens* is usually a meat or meat sauce or gravy. In this series of experiments, a formula for chicken gravy designed by Felstehausen (1962) was selected to serve as the culture and freezing medium. The gravy was prepared as follows:

90 g	chicken fat
125 g	flour
120 g	instant non-fat dry milk
9 g	salt
1350 ml	water
200 g	cooked chicken meat

Stewing hens weighing approximately 5 to 6 pounds, purchased in the local market, were used as the source of chicken fat and meat. The chickens were washed, cooked in an autoclave at 121°C at 15 psi for 45 minutes. The fat was recovered, and the chickens were deboned. Ninety grams of fat were weighed into each of several sterile jars and the jars sealed. The chicken meat in 200-g portions was put into plastic bags. The jars and bags were immediately put into a freezer which maintained a temperature of −17.7°C.

In preparing the gravy, 75 g of flour were added to the melted fat. The mixture was cooked until it became smooth. The remainder of the flour was added to 250 ml cold water to form a smooth paste. The paste was gradually added to the fat-flour mixture. The dry milk was reconstituted with the remaining 1100 ml water and added gradually to the gravy, stirring constantly. Salt and chicken pieces were added. The gravy mixture was then cooked over medium heat with stirring until thick—about 10–15 min. Portions of the prepared chicken gravy were placed in ½-pint glass jars, which were sealed and then autoclaved for 25 min at 15 psi.

[a] Published with the permission of the Director of the Wisconsin Agricultural Experiment Station, Madison.

Prior to use, each sample of gravy was aseptically mixed to ensure equal distribution of the meat particles and chicken fat. Approximately 20 ml of the gravy was transferred to a sterile screw-cap vial (capacity 30 ml). The gravy in the vial was then inoculated with 1 ml of a 16-hr culture of *C. perfringens* which had been grown in fluid thioglycollate with sugar (Difco). The chicken gravy culture was then allowed to incubate 6 hr at 37°C. After incubation, 1 ml of the culture was diluted in phosphate buffer (American Public Health Association, 1960) and plated in duplicate. The plating medium was SPS agar, as described by Angelotti *et al.* (1962) except that both polymyxin B sulfate and sodium sulfadiazine were omitted. From this plating, a population of the chicken gravy culture was obtained prior to freezing. Simultaneously, 1-ml portions of the culture were placed with a cannular syringe into each of 12 sterile sections of soft glass tubing (six mm OD, approximately 15 cm long) which had been constricted, but not sealed, at one end. After the chicken gravy culture was placed in the tube, both ends of the tubing were sealed with a flame. The tubes were then immersed in ethyl alcohol at −17.7°C and held at this temperature.

At the completion of a desired holding time, the sealed glass tube was removed from the alcohol, dried, and aseptically broken in a tube containing 9 ml of phosphate buffer. After minimum agitation to ensure thorough mixing, a 1-ml portion was further diluted and then plated in duplicate to determine the population of the original chicken gravy culture that had survived freezing. The plates were incubated at 37°C in an atmosphere of 90% nitrogen and 10% CO_2. Black colonies are produced in this medium by *C. perfringens*. These colonies were observed and counted after both 24-hr and 48-hr incubations. Populations of the chicken gravy, both prior and following holding at freezing temperatures, were compared in this manner.

Five type A strains of the organism were used: 8799F 1546/52, isolated from food incriminated in a food poisoning incident in England and furnished through the courtesy of Dr. B. C. Hobbs; 65, isolated from raw carrots; 108, isolated from raw veal liver; 142A, isolated from raw ground beef; and 214D, isolated from the feces of a victim in a food poisoning incident and supplied by Dr. E. R. Krumbiegel.

The holding times selected to compare survival rates with that of the original chicken gravy culture populations were: 1, 2, 3, 10, 20, 30, 60, 90, 120, 150, and 180 days. The procedure was repeated 4 times.

Series II. Similar experiments were undertaken with a spore-soil and chicken gravy mixture instead of utilizing growth of *C. perfringens* in chicken gravy. Spores were obtained by incubating 1 ml of an active thioglycollate without sugar (Difco) culture for 22 hr in a sporulating medium described by Ellner (1956). Two ml of the Ellner's medium culture were then pipetted onto approximately 10 g of sterile dry soil and enclosed within a screw-cap vial. The spore suspension was allowed to dry on the soil medium over a period of several weeks. The soil was then mixed to ensure a finely divided, uniform source of inoculum. This spore-soil inoculum was used for each of the four replicates over a period of one month.

Approximately ½ g of the spore-soil inoculum was mixed with 20 ml of the chicken gravy. Some samples of the gravy, inoculated with the spores, were plated immediately to secure initial counts; others were placed in glass tubing and frozen and treated as was the gravy culture in Series I. Four of the 5 strains used in Series I were used in this series of experiments. The fifth strain sporulated poorly in Ellner's medium. In this series, observations on the second and twentieth days of holding were eliminated. The freezing temperature was identical in both series of experiments.

Since in both series of experiments the percentage survival of cells and spores varied widely among the four replications, Torrie (1963) recommended a procedure to be used in drawing averages which would minimize the effect of large values. He proposed that the log of the percent survival for each replication be found, and that an average log value be ascertained; the antilog of the average log would express the percentage survival of the population. This procedure was followed.

RESULTS AND DISCUSSION

Tables 1 and 2 present results representative of the trends observed. Table 1 indicates that in most cases a large percentage of the population of *C. perfringens* grown in chicken gravy was not recovered after the initial freezing and subsequent thawing. It should be pointed out that the range of plate counts, considering all replicates from which the average percentage survivals were derived, was rather wide, but in no case were any plates totally devoid of colonies. There is no apparent explanation for the behavior of Strain 214D. Some of the variation may have been caused by the lack of homogeneity of the chicken gravy which served as the suspending medium. Except in the one case, less than 20% of the cells were recovered after being held frozen for 1 day. Apparently, with some fluctuation, this residual

Table 1. Survival of *C. perfringens*[a] grown in chicken gravy for 6 hr followed by freezing, holding for various periods at −17.7°C, and thawing.

Strain	Origin	Av. initial count (cells/ml)	Calculated percentage[b] survival of cells at: 1 day	10 days	30 days	90 days	180 days
8799F	Food in food-poisoning outbreak	1.38×10^{8}	×8.05	×11.30	×2.90	1.85	3.69
1546/52							
65	Raw carrots	1.64×10^{7}	17.11	3.94	3.04	1.03	0.12
108	Raw veal liver	1.53×10^{7}	9.12	4.84	6.51	3.82	0.02
142A	Raw ground beef	1.74×10^{7}	14.36	4.22	1.15	2.07	×0.03
214D	Feces—food-poisoning outbreak	1.75×10^{8}	224.30	17.94	5.38	4.29	0.95

[a] Inoculum was a 16-hr culture grown in thioglycollate medium with sugar.

[b] Values presented are derived from dual platings in each of 3 or 4 replications (numbers marked × from 3 replications; others from 4). The percentage figures were derived by obtaining the log expression for each percentage survival calculated from the observations, averaging the logs, and accepting the antilog of the average as the percent survival.

Table 2. Survival after freezing, holding for various periods at −17.7°C, and thawing *C. perfringens* added to chicken gravy as a spore-soil mixture.

Strain	Av. initial count (cells/ml)	Calculated percentage[a] survival of cells at: 1 day	10 days	30 days	90 days	180 days
65	5.98×10^{4}	50.6	93.2	97.8	33.6	10.9
108	6.24×10^{4}	13.7	87.7	58.6	17.4	5.4
142A	5.00×10^{4}	48.3	76.5	63.6	37.9	2.2
214D	3.03×10^{4}	90.2	154.1	33.4	16.4	6.0

[a] Values presented are derived from dual platings in each of 4 replications. The percentage figures were derived by obtaining the log expression for each percentage survival calculated from the observations, averaging the logs, and accepting the antilog of the average as the percent survival.

population gradually diminished until the 180th day, when the over-all average value indicated that about 1 percent of the cells were recovered.

When the spore-soil inoculum was added to the gravy and frozen, the percentage survival appeared to be relatively high for 30 days (Table 2). Thereafter, the decline to 180 days was fairly rapid, but even then 2–10% of the population was viable. Under the conditions of these experiments, not all spores were killed.

When the effect of −17.7°C on an organism in a food mixture is evaluated, the question arises whether the situation in the laboratory is typical of a practical situation. In this instance, it is difficult to know whether cells grown in the food or spores added to the foods are more directly comparable to the organisms which would develop when a food becomes infected during preparation or processing. It is probable that the population of *C. perfringens* produced by growing the cells in the gravy for 6 hr was largely in the vegetative state. Essentially then, the observed difference between cells grown in the gravy and spores added to the gravy was caused by the phase of cell development. In these experiments, a difference in size of population existed which cannot be overlooked as an influencing factor in determining survival rate.

Other factors meriting consideration include the nature of the medium on which the spores were produced and the means used to recover the cells, which were possibly damaged by freezing and thawing. Cash and Collee (1962) described experiments in which Ellner's medium was used as the medium of choice in attempting, for one strain of *C. welchii (perifringens)*, to correlate sporulation and the development of resistance to certain stresses. In 1963, however, Hall *et al.* concluded that spores produced on Ellner's medium were "atypical" and differed from spores produced on certain other media. It is conceivable that spores produced in Ellner's medium may when

frozen react differently from spores grown in other media.

In recovering *C. perfringens*, aside from the omission of polymyxin B sulfate and sodium sulfadiazine from the selective medium, no other procedure was employed to compensate for the possibly more exacting growth requirements of frozen and thawed cells. Research workers have found that, for certain other organisms, the recovery of frozen and thawed cells increased when special thawing agents and diluents were employed (Bretz and Hartsell, 1959). Greater accuracy would perhaps be achieved in the recovery of *C. perfringens* if similar techniques were developed.

Assuming that *C. perfringens* incubated in chicken gravy for six hours produced primarily a vegetative cell population, the implication exists that vegetative cells as well as spores may, under certain conditions, survive freezing at −17.7°C for 180 days. If food-poisoning strains of *C. perfringens* perform similarly to the strains tested here, the possibility exists that some frozen foods, if mishandled, may be a health hazard as a result of the presence of this organism.

ACKNOWLEDGMENTS

This investigation was supported in part by Public Health Research Grant EF-131(C1) from the Division of Environmental Engineering and Food Protection.

Appreciation is expressed to Dr. Betty C. Hobbs and Dr. E. R. Krumbiegel for cultures used in these studies.

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The Determination of the Tensile Strength of Fluid Food Materials

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(Manuscript received April 16, 1963)

SUMMARY

The tensile strength of fluid food materials is a rheological property which is rarely considered, although it may play a role in texture and coating behavior in certain cases. It is possible to measure the tensile strength of the fluid by forcing it slowly downward through a vertical tube which is less than the critical diameter. The fluid column will break when the weight of the column divided by the cross-sectional area of the column equals the tensile strength of the fluid. The tensile strength of ketchup, tomato paste, and mayonnaise was measured and found to be about twice the shear strength. Tube diameter had no effect on measured tensile strength provided it was less than the critical diameter.

INTRODUCTION

The structural strength of a food material determines the effect of processing and is an indication of texture. Among the properties that determine the structural strength of material is the tensile strength.

The tensile strength refers to the force/area required to separate material into planes perpendicular to the line of force. Shear strength, on the other hand, refers to force/area required to move one layer of material past another in planes that are parallel to the line of force.

The tensile strength of fluid food materials is a property that is rarely considered. It is true that, under most flow conditions, the shear forces are the most prominent forces, and for most fluids the shear strength is considerably less than the tensile strength. For example, although the shear strength of water is small enough to defy measurement, the tensile strength of water is theoretically greater than that of steel (Harkins, 1926). Thus, under most flow conditions, fluids yield in shear rather than tension.

There are situations, however, where the tensile properties may play as great a role as the shear properties of the fluid. For example, the "texture" of fluid foods and the coating behavior of fluids under certain conditions may be affected by their tensile properties.

The tensile strength of fluid food materials may be measured with a relatively simple system.

DETERMINATION OF FLUID TENSILE STRENGTH

Fluid tensile strength may be determined by forcing a fluid slowly down through a vertical tube with a diameter small enough to prevent flow unless pressure is applied (see Fig. 1a). Under these conditions, the fluid column will break periodically outside the tube. The break occurs near the mouth of the tube when the weight of the column outside the tube divided by the cross-sectional area of the column equals the tensile strength of the fluid food material.

Therefore, by measuring the weight of material associated with the periodic breaks and the cross section of the column, the tensile strength may he determined from

$$S_t = \frac{W}{A} \qquad [1]$$

where:

S_t = tensile strength of material
W = weight of material
A = cross-sectional area of column at breaking point

The critical diameter of the tube that will

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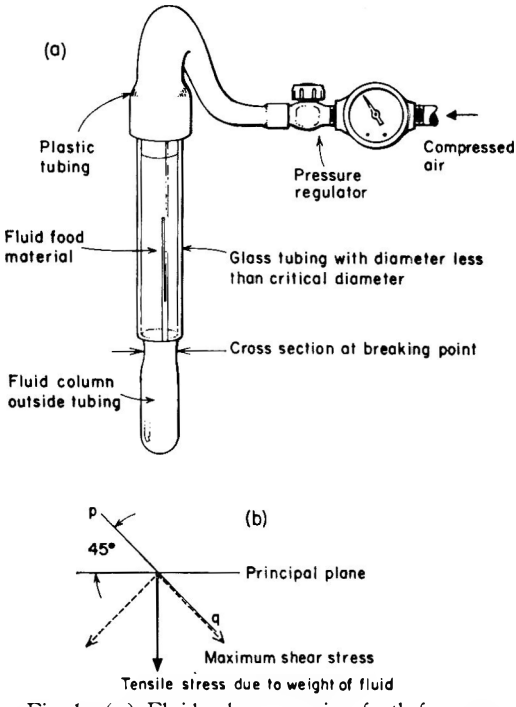

Fig. 1. (a) Fluid column moving forth from supporting tube; (b) Stresses acting on a plane inclined 45° to principal plane.

not permit flow without applied pressure may be calculated by noting that the fluid weight in the tube must be supported by the shear strength of the fluid acting along tube wall, or

$$G = C\,2\pi RL = \mathrm{P}\,\pi\,R^2\,L \qquad [2]$$

or

$$R = \frac{2C}{\mathrm{P}}$$

where:

C = shear strength of material
G = weight of fluid in the tube
P = density of the fluid
L = length of tube in contact with fluid
R = the critical radius

It is interesting to note that a vertical tube with a critical radius will hold up any height of fluid unless additional pressure is applied.

SHEAR STRENGTH DETERMINATION

Methods for determining the shear strength of fluids have been discussed in detail elsewhere (Charm, 1963). The shear strength may be measured by noting torque on a serrated cylinder that has come to rest in a fluid, or by employing Casson's equation, i.e., plotting (shear stress)$^{1/2}$ vs. (shear rate)$^{1/2}$ and extrapolating to zero shear rate or by noting the weight remaining on a vertical plate with a rough surface that has been dipped in the fluid, removed, and allowed to drain.

EXPERIMENTAL PROCEDURE

The tensile strengths of Kraft mayonnaise and Heinz ketchup were determined by drawing the fluids up in a glass tube 0.4 cm in diameter. This tube diameter is less than the critical diameter for the fluids considered and was conveniently available. The glass tube was then held in a vertical position and connected to a compressed-air supply. The compressed air slowly forced the fluid from the tube (see Fig. 1a).

The fluid moved forth from the tube and reached a critical length causing a break in the fluid near the mouth of the tube. The diameter of the column prior to breaking was measured with dividers and a magnifying glass, and the fluid that separated from the column was weighed. There is about a 15% error associated with the method of measuring the cross-sectional area of the column at the breaking point. Care must be exercised to avoid air bubbles in the column. This is the primary cause of disagreement between replicates. Employing Eq. 1, the tensile strength was calculated (see Table 1). The effect of tube diameter on tensile strength was determined using diameters of 0.4 cm and 0.6 cm with Hellmann's mayonnaise and Hunt's tomato paste (see Table 2).

The shear strength of the fluids may be determined by dipping a thin plate, 2 × 2 inches, with a rough surface in the fluids and noting how much material remains after removing the plate vertically from the fluid (Charm, 1962). The shear strength is calculated using this procedure from the following equation:

$$C = \frac{W}{A} \qquad [3]$$

where:

W = weight remaining on plate
A = surface area associated with the fluid

There is about a 10% error associated with this method (see Table 3).

The yield stress may also be determined by measuring the torque on a serrated cylinder that has been allowed to come to rest in the fluid. In this case, a Haake concentric-cylinder viscometer equipped with a serrated bob was employed (Charm, 1963).

The shear strength or yield stress of mayonnaise determined by this method is 580 dynes/cm^2, compared with an average of 669 dynes/cm^2 determined with the vertical-plate method. In this range there is about a 10% error associated with determining shear strength by this method.

Table 1. Tensile strength determined by weight required to break vertical column.

Material	Weight of fluid breaking column (dynes)	Cross section of column (cm^2)	Tensile strength $\left(\frac{dynes}{cm^2}\right)$
Ketchup	(.11) (980)	0.1	1070
(Heinz)	(.10) (980)	0.1	980
	(.09) (980)	0.1	870
	(.10) (980)	0.1	980
	(.085) (980)	0.1	835
Mayonnaise	(.15) (980)	0.1	1470
(Kraft)	(.18) (980)	0.1	1760
	(.17) (980)	0.1	1660
	(.19) (980)	0.1	1860
	(.15) (980)	0.1	1470

Table 2. Comparison of tensile strengths determined in different tube diameters.

Material	Tube diam. (cm)	Cross-sectional area of column (cm^2)	Tensile strength $\left(\frac{dynes}{cm^2}\right)$
Mayonnaise			
(Hellmann's)	0.40	0.10	2730
			2680
	0.60	0.25	2680
			2630
Tomato paste	0.40	0.10	2650
(Hunt's)			2730
	0.60	0.25	2640
			2660

Table 3. Shear strength determined by weight remaining on a vertical plate.

Material	Weight remaining on vertical plate (dynes)	Plate area in contact with fluid (cm^2)	Shear strength $\left(\frac{dynes}{cm^2}\right)$
Ketchup	(8.97) (980)	20.3	434
(Heinz)	(8.60) (980)	20.3	415
	(8.70) (980)	20.3	420
	(9.30) (980)	20.3	448
	(8.70) (980)	20.3	420
Mayonnaise	(14.25) (980)	20.3	686
(Kraft)	(13.60) (980)	20.3	655
	(15.28) (980)	20.3	738
	(13.70) (980)	20.3	665
	(12.48) (980)	20.3	602

DISCUSSION OF RESULTS

The tensile strengths of the mayonnaise and ketchup are about 2 to 2½ times as great as the shear strengths. The reason may be deduced by considering the stresses acting on the plane inclined 45° to the principal plane (see Fig. 1b).

The stress caused by W acting on the inclined plane, pq, may be resolved into two components, one perpendicular to the inclined plane and one parallel to the inclined plane. The component acting parallel to the inclined plane, pq, is a shear stress. It has been shown (e.g., Timoshenko and MacCullough, 1949) that the shear stress acting on the plane inclined 45° to the principal plane is the maximum shear stress set up by the tensile stress and is equal to ½ the tensile stress acting on the principal plane of the body. Wherever a shear stress occurs that exceeds the shear strength, the body yields.

Thus, when the tensile stress in the column of fluid is great enough to set up a shear stress that exceeds the shear strength on the plane inclined 45° to the principal plane, the body will break. The tensile stress on the principal plane will theoretically be twice the shear stress on the plane inclined 45° to the principal plane.

In another example of this effect, Timoshenko and MacCullough (1949) noted that if a bar of mild steel with a polished surface is loaded in tension until the yield point is reached, then the yield of the metal is visible to the naked eye and the yielding begins along those inclined planes for which the shearing stress approaches a maximum. These planes of sliding are revealed on the surface by easily discernible lines, which are called Lueder's lines.

In our experiment it could not be determined visually that, with mayonnaise or ketchup, yielding actually commenced along the inclined planes. However, the relationship of the tensile strengths of mayonnaise and ketchup to the shear strengths suggest that this is occurring. If this is the case, the tensile strength determined does indeed specify how the mayonnaise or ketchup will yield when loaded with a tensile stress, but it does not specify the stress required to separate molecules in the same layer.

In the experimental determination of tensile strength, it is noted that the diameter of the fluid column is smaller than the tube diameter. This suggests that the fluids

undergo "strain hardening"; that is, the weight of the column stretches the fluid, causing a decrease in cross-sectional area but at the same time an increase in tensile strength due to molecular rearrangement. Eventually, the column is stretched to the point where the increase in tensile strength due to stretching no longer offsets the decrease in cross-sectional area, and the column breaks. "Necking," or the distortion of the cross section to a very narrow section at the point of the break, is evidence of strain hardening (Reiner, 1960).

The tensile strength of the fluid is independent of the tube diameter in which the measurement is made, as it theoretically should be (see Table 2).

It appears that, for a more complete description of the rheological behavior of fluid food materials, their tensile strength along with their shear strength and viscometry should be considered.

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Relation of Hexanal in Vapor Above Stored Potato Granules to Subjective Flavor Estimates

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(Manuscript received October 18, 1963)

SUMMARY

Influence of air storage of commercial potato granules at 22°C was determined by gas chromatography and flavor tests by judges. Increases in hexanal content of the vapor above the reconstituted product at 93°C followed subjective flavor estimates closely. Sixty-five percent of the judges distinguished between air-packed granules and a control sample packaged in nitrogen atmosphere after approximately three months (5% level of significance). During this time, hexanal concentration above the reconstituted air-packed granules increased from 0.2 to 2.5 ppm. By the end of the fourth month, when hexanal had increased to 8.5 ppm, almost all of the judges detected flavor deterioration.

INTRODUCTION

Oxidative deterioration, sometimes referred to as rancidity, is the predominant change in potato granules stored in air atmosphere at moderate temperatures (Hendel, 1959). Hexanal is a major product of autoxidation of linoleic acid and is commonly present in autoxidized oils and fats. The present study uses the relatively new direct vapor-injection gas chromatography method in following hexanal changes with air-packed potato granules stored at 22°C, and it relates these changes to subjective estimates of flavor changes during storage.

MATERIALS

Commercially processed potato granules were obtained from an Idaho processor in March, 1962. They contained 7% moisture, 2.5 ppm of butylated hydroxytoluene (BHT), and 250 ppm of sulfite as SO_2. The material as received was packaged in nitrogen in no. 10 cans. The cans were opened, and all of the material was mixed thoroughly in a stainless-steel container and then repackaged in 6-oz cans (70 g/can). Half of the cans were sealed in air and the other half in nitrogen. Average oxygen content of the nitrogen packs was 1.4%, with a standard deviation of 0.2. All cans were stored at 22°C.

CHROMATOGRAPHY METHOD

The gas-liquid chromatography (GLC) apparatus was the dual-flame ionization detector type (Buttery, 1961; Buttery and Teranishi, 1961, 1963). The packed columns were 5 ft long by 0.25 in. OD and 0.21 in. ID stainless-steel packed with a 30% Apiezon M on 60–80-mesh firebrick. Column temperature was 115±0.5°C. The column carrier nitrogen flow was 30 ml per minute.

Fifteen grams of potato granules were added to 150 ml of boiling, distilled water in a 250-ml Erlenmeyer flask. The flask was immediately covered with aluminum foil, and the mixture was gently swirled for exactly 60 seconds; temperature of the mix was then 93°C. At this point, a room-temperature hypodermic syringe with needle removed was pushed through the foil, and a 10.0-ml sample of the vapor taken with the end of the syringe about 1 in. above the surface of the mixture. The 25-gauge needle was quickly replaced, and the sample was injected into the GLC unit. Usually, 3 cans of granules per storage time were analyzed and the results averaged.

The concentration of hexanal in the vapor (ppm or g of hexanal/10^6 g of air) at the time and temperature of sampling was calculated from peak area using a calibration factor found by injection

[a] Collaborator employed by the Instant Potato Granule Mfg. Assoc.

[b] Reference to a company or product does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

of known amounts of benzene. Detector response to hexanal was assumed to be the same as for benzene.

SUBJECTIVE FLAVOR METHOD

The granules were appraised as mashed potatoes. Two hundred and forty ml of distilled water containing 1 g of NaCl was heated to 71°C, 50 g of granules was added, and the mixture was stirred with a fork for 60 strokes in 30 seconds. The reconstituted mix was held during appraisal periods in a water bath thermostatically controlled at 71°C.

The reconstituted granules were evaluated by 20 trained selected judges in booths lighted by 7.5-watt green globes to mask possible appearance differences. A control sample which was nitrogen-packed and stored at 22°C was compared by duo-trio tests with each of a series of samples held for various times in air at the same temperature. At least two replicate duo-trios were tested at each storage time by the 20 judges, giving at least 40 judgments per storage time. The question to judges was, "Which of the two coded samples tastes more like the labeled sample?" Judges knew that the labeled sample was a duplicate of one of the coded samples. As differences between the coded samples increase with increasing storage time, more judges are able to match the duplicate samples.

Usually, the control sample in our potato granule storage experiments is nitrogen-packed and stored at −34°C (Buttery *et al.*, 1961). Under these conditions, no measurable flavor change occurs up to at least one year. In the present experiment the nitrogen-packed control granules were stored at 22°C instead of −34°C, in order to limit the flavor difference between the control and test samples primarily to oxidative off-flavor. Hendel (1959) has reported that a small amount of non-enzymatic browning occurs at 22°C, but the rate of the reaction is approximately the same in air and nitrogen atmospheres. Very little oxidation would occur in the nitrogen-packed control sample, but this reaction would proceed rapidly in the air-packed test granules. Thus, the samples submitted to judges would differ primarily in oxidative off-flavor.

RESULTS AND DISCUSSION

When potato granules were air-packed and stored at 22°C, hexanal concentration in the vapor above the reconstituted product at 93°C increased with time of storage (Fig. 1), but rate of change was not uniform. The shape of the curve indicates an induction period followed by rapid change; this is typical of fat oxidation. During the

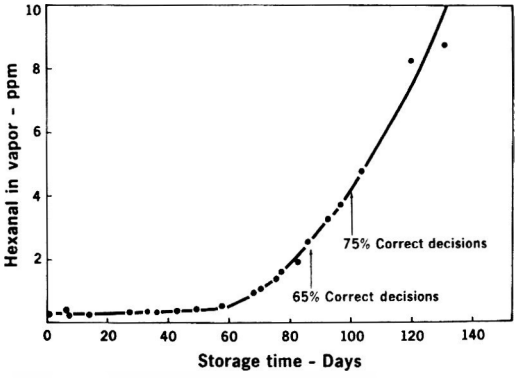

Fig. 1. Hexanal concentration in the vapor above reconstituted potato granules (at 93°C) which had been air-packed and stored at 22°C.

first 60 days, hexanal concentration in the vapor increased from approximately 0.2 to only 0.6 ppm, by 80 days to 1.6 ppm, and thereafter the rate of increase was of the order 0.15 ppm per day, the concentration after 125 days being 8.5 ppm.

A few judges consistently distinguished between air-packed and nitrogen-packed granules after short periods (Fig. 2), and

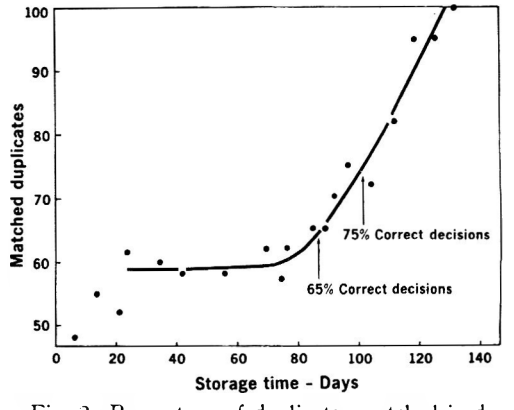

Fig. 2. Percentage of duplicates matched in duo-trio tests for comparisons of air- and nitrogen-packed potato granules stored at 22°C (20 judges; at least 2 replicate duo-trios per judge for each storage period).

65% of the panel matched duplicate samples when the granules had been stored for 87 days (significant at the 5% level). At this time, hexanal concentration for the air-packed sample had increased from 0.2 to 2.5 ppm. According to the 75%-correct-decision criterion which we report in all storage study publications, off-flavor developed after 101 days when hexanal concentration was 4.2 ppm. By the end of the fourth month,

when hexanal had increased to 8.5 ppm, almost all of the judges detected deterioration in all replications submitted to them. The judges' data exhibit considerable scatter of points, which is probably due to the sulfite, BTH, and browning present in all samples. Figs. 1 and 2 clearly show that hexanal concentration was closely associated with flavor deterioration of potato granules under the conditions of the investigation.

Although hexanal was used here as an index of rancidification, we do not intend to imply that it is mainly responsible for the characteristic rancid flavor of granules. Rancidity is probably a function of several compounds produced in fatty acid autoxidation. Other autoxidation compounds could be followed in the same way as was done here with hexanal.

ACKNOWLEDGMENT

The writers thank the tasters for their cooperation in carrying out this project.

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Taste Interrelationships. VI. Sucrose, Sodium Chloride, and Citric Acid in Canned Tomato Juice

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(Manuscript received November 16, 1963)

SUMMARY

Apparent sweetness, saltiness, and sourness in canned tomato juice was evaluated by trained judges categorized according to three levels of sensitivity. A method of paired-comparison constant-stimulus was used in which judges indicated the direction of the response as well as the magnitude of difference in taste intensity within each pair. The apparent sourness of citric acid was depressed by both sucrose and sodium chloride. The sweetness of sucrose was reduced by citric acid, but enhanced by low levels of sodium chloride. Although the saltiness of sodium chloride was reduced by sucrose, it was significantly enhanced by acid. In the latter combination of taste stimuli, variability between judges was exceptionally large. Frequency of reversal of direction of response between duplicate pairs was greater among judges of low than among judges of high sensitivity. The extent to which the results agree with previous relationships with aqueous media and lima bean purée is discussed.

INTRODUCTION

Extensive investigations have been made on the interaction of the basic tastes in solutions of distilled water (Fabian and Blum, 1943; Kamenetzky and Pilgrim, 1958; Beebe-Center *et al.*, 1959; Kamen *et al.*, 1961; Pangborn, 1960, 1961, 1962). Pangborn and Trabue (1964a) recently studied the taste interrelationship of sucrose, sodium chloride, and citric acid in lima bean purée and reported good agreement of responses with those established in distilled water.

In the present investigation, the main objective was to intercompare the apparent taste intensities of sucrose, sodium chloride, and citric acid in a natural food product which normally elicits sweetness, saltiness, and sourness—tomato juice. In addition, the effect of the original taste sensitivity of the experimental subjects on their subsequent responses was measured.

METHODS AND MATERIALS

Panel selection. A method of sequential analysis described by Amerine *et al.* (1959) was used to select 13 judges from 21 people tested. On each of 18 days of testing, each judge received 6 randomly presented triangle tests of commercially canned tomato juice, two varying in amount of added sucrose, two in amount of added sodium chloride, and two in amount of added citric acid. The judge's task was to designate the odd sample within each triangle set and to indicate whether the odd sample or the duplicates contained the greater intensity of the taste under study. The thirteen judges who fell within the region of acceptance showed a further separation into three distinct subgroups, which we will refer to subsequently as high (85.7–95.2% correct), medium (71.4–77.8%), and low (59.3–61.9%) sensitivity.

Experimental method. Used throughout the six phases of the investigation was a method of paired-comparison constant-stimulus described in detail previously (Pangborn, 1961). Each judge received four pairs in duplicate, for a total of eight randomly presented pairs per session. Within a pair, both members contained the secondary taste compound whereas only one contained the primary compound. Judges were asked to circle the number of the sample within each pair which contained the greater apparent intensity of the secondary taste sensation. In addition, the degree of difference within a pair was indicated on a scale labeled slight, moderate, large, or extreme. These descriptive terms were converted to integers of −1, −3, −5, and −7 if the sample without the primary compound was selected, and +1, +3, +5, and +7 if the sample with the primary compound was selected as more intense. These difference scores were then submitted to analysis of variance. Chi-square analysis was applied to determine the significance of difference in the paired selections.

Sample preparation and presentation. The tomato juice was prepared with no additives, from

Red Top variety tomatoes in the pilot plant of the Department of Food Science and Technology in September, 1961. The cans were stored at 40°F for 13 mo prior to initiation of the present study. The pH, total acidity, and refractive index of the juice were 4.4, 0.292%, and 1.3412. Throughout the investigation, pH, total acidity, and refractive index were measured for each experimental sample to assure accuracy of preparation. Concentrations of sucrose, sodium chloride, and citric acid were selected by thorough screening, to range from threshold to very intense for the specific combinations of tastes. The exact concentrations used are shown in Table 1 and in Figs. 1–6.

Samples were prepared on alternate days and stored in covered glass containers at 36°F. Reagent-grade sucrose and sodium chloride were added to the juice, w/v. The citric acid was first made into a 50% stock solution from which measured volumes were pipetted into measured volumes of juice. To compensate for dilution, equal quantities of distilled water (e.g. 1.7 ml water/1700 ml juice) were added to samples compared against those containing the citric acid.

The tomato juice was served daily between 9:00 and 10:00 A.M. at room temperature (70±2°F) under low, red illumination. Approximately 35 ml of sample was tested. Prior to tasting the experimental set, judges tasted a "warm-up" sample to orient them to the compounds being evaluated on that day. Instructions were given to refrain from swallowing any juice or distilled water, but to use distilled water for oral rinsing between pairs. Immediately after tasting, judges were provided with the results and a "reward" of cookies, crackers, or coffee cake.

Within a study, the order in which the treatments were served, the order of the pairs within a set, and the samples within a pair were randomized. Judges unable to attend a testing period were required to taste a "make-up" the following day, so that all samples were tested by all judges an equal number of times. As indicated in Table 5, only one judge was unable to complete each of the six studies. After a two-week break for the Christmas holidays, two days of reorientation were held before the presentation of experimental samples was resumed.

RESULTS

Study I. Effect of sucrose on apparent sourness of citric acid. The frequency of selection of the sample with the greater apparent sourness (Table 1) and the plotted difference scores (Fig. 1) show that sucrose depressed apparent sourness. The sourness of the higher levels of citric acid was depressed more than was the lowest level (0.01%). Analysis of variance of the difference scores

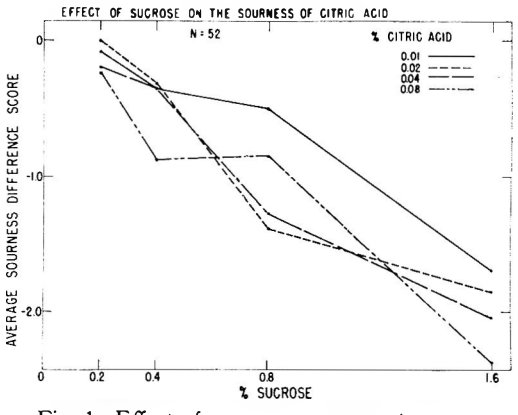

Fig. 1. Effect of sucrose on apparent sourness of citric acid in canned tomato juice.

showed no significant variation attributable to citric acid. The very highly significant difference between judges, between groups, and between judges within groups attests to the lack of agreement between individual judges in intensity of difference. Lack of significance due to replications and interactions involving replications emphasizes the reproducibility of response within the panel and within a judge. All three groups of judges agreed that sucrose depressed sourness, with judges 7 and 12 indicating slight positive average scores (Table 5).

Study II. Effect of citric acid on apparent sweetness of sucrose. Levels of citric acid between 0.04 and 0.16% significantly depressed the sweetness of 0.2 to 1.6% sucrose (Table 1, Fig. 2). Judges

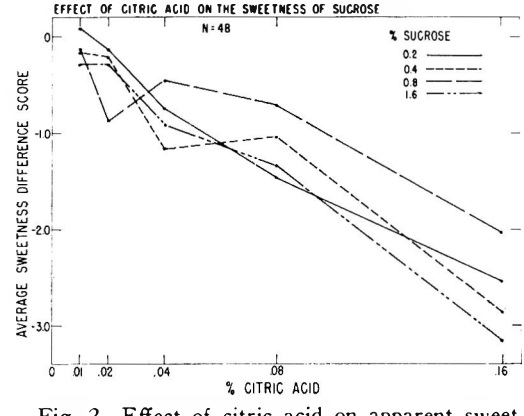

Fig. 2. Effect of citric acid on apparent sweetness of sucrose in canned tomato juice.

were in complete agreement on direction of response even though opinion of intensity of difference varied. Once again, judges and interactions involving judges were significant, whereas replications were not (Table 2). Groups of judges contributed significantly to the variation, with

Table 1. Taste intensity of sucrose, sodium chloride, and citric acid in canned tc intense within each pair.

I. Sucrose-sourness (n = 52)
Primary:

Sucrose (%)	0.2	0.4	0.8	1.6	Total
% Citric					
0.01	26	32	36[b]	43[c]	137[c]
0.02	30	36[b]	43[c]	45[c]	154[c]
0.04	30	31	41[c]	46[c]	148[c]
0.08	30	40[c]	41[c]	49[c]	160[c]
Total	116	139[c]	161[c]	183[c]	599[c]

III. Sucrose-saltiness (n = 52)
Primary:

Sucrose (%)	0.3	0.6	1.2	2.4	Total
% NaCl					
0.2	32	29	33	38[c]	132[c]
0.4	26	33	31	37[b]	127[b]
0.8	33	36[b]	45[c]	44[c]	158[c]
1.6	34[a]	33	43[c]	44[c]	154[c]
Total	125[b]	131[c]	152[c]	163[c]	571[c]

V. Citric-saltiness (n = 48)
Primary:

Citric acid (%)	0.015	0.045	0.135	Total
% NaCl				
0.16	20	19	13[b]	52[c]
0.48	29	17	5[c]	51[c]
1.44	17	11[c]	8[c]	36[c]
Total	66	47[c]	26[c]	139[c]

[a, b, c] Significant difference within pairs at p = 0.05, 0.01, and 0.001 respectively.

mato juice. Frequency of selection of sample without primary as most

II. Citric-sweetness (n = 48) Primary: Citric acid (%)	0.01	0.02	0.04	0.08	0.16	Total
% Sucrose						
0.2	22	29	35[b]	41[c]	45[c]	172[c]
0.4	29	28	38[c]	40[c]	47[c]	182[c]
0.8	27	38[c]	31	35[b]	41[c]	172[c]
1.6	28	27	35[b]	37[c]	48[c]	175[c]
Total	106	122[c]	139[c]	153[c]	181[c]	701[c]

IV. Sodium chloride-sweetness (n = 52) Primary: NaCl (%)	0.05	0.1	0.2	0.4	0.8	1.6	Total
% Sucrose							
0.2	11[c]	10[c]	5[c]	14[c]	21	31	92[c]
0.4	13[c]	6[c]	8[c]	12[c]	21	31	91[c]
0.8	19	11[c]	7[c]	8[c]	19	31	95[c]
1.6	12[c]	11[c]	8[c]	19	22	34[a]	106[c]
Total	55[c]	38[c]	28[c]	53[c]	83[b]	127[b]	384[c]

VI. Sodium chloride-sourness (n = 52) Primary: NaCl (%)	0.02	0.04	0.08	0.16	0.32	Total
% Citric						
0.01	28	39[c]	42[c]	39[c]	43[c]	191[c]
0.02	35[a]	33	44[c]	43[c]	42[c]	197[c]
0.04	36[b]	42[c]	41[c]	41[c]	45[c]	205[c]
0.08	26	35[a]	41[c]	39[c]	44[c]	185[c]
Total	125[b]	149[c]	168[c]	162[c]	174[c]	778[c]

Table 2. Analysis of variance of difference scores. Interaction of sucrose and citric acid.

Source of variation	I. Sucrose-sourness [a]		II. Citric-sweetness [b]	
	Degrees of freedom	Mean square	Degrees of freedom	Mean square
(S) Sucrose	3	140.02***	3	5.85
(C) Citric acid	3	7.01	4	189.43***
(R) Replications	3	4.34	3	5.85
(J) Judges	12	31.06***	11	26.86***
(G) Groups	2	82.43***	2	32.64***
(Y) Judges within groups	10	20.78***	9	25.58***
S × C	9	3.67	12	5.42**
S × R	9	3.02	9	1.19
S × J	36	3.99	33	3.68*
G × S	6	1.11	6	3.04
Y × S	30	4.57*	27	3.83*
C × R	9	3.04	12	1.33
C × J	36	4.38*	44	8.73***
G × C	6	3.05	8	12.63***
Y × C	30	4.65*	36	7.86***
R × J	36	2.35	33	2.07
G × R	6	1.02	6	10.29***
Y × R	30	2.62	27	0.24
S × C × R	27	2.46	36	3.15
S × C × J	108	3.39	132	5.24**
S × R × J	108	2.47	99	3.72**
C × R × J	108	1.79	132	2.91
Remainder [c]	324	3.01	396	2.34

[a] Effect of sucrose on sourness of citric acid.
[b] Effect of citric acid on sweetness of sucrose.
[c] Error term (S×C×R×J).
*, **, *** Respectively significant at p = 0.05, 0.01, and 0.001.

Group I assigning the highest negative average difference score, followed by Groups II and III (Table 5).

Study III. Effect of sucrose on apparent saltiness of sodium chloride. With increasing additions of sucrose up to 2.4%, the saltiness of 0.8 and 1.6% sodium chloride was greatly depressed, whereas that of 0.2 and 0.4% was slightly lowered (Table 1, Fig. 3). That the saltiness of the different levels of sodium chloride was affected differently is verified by the analysis of variance (Table 3). Less variation was attributable to judges in this study than in the other five studies, for all but one judge assigned negative scores (Table 5). Neither replications nor groups of judges contributed significantly to the observed variation.

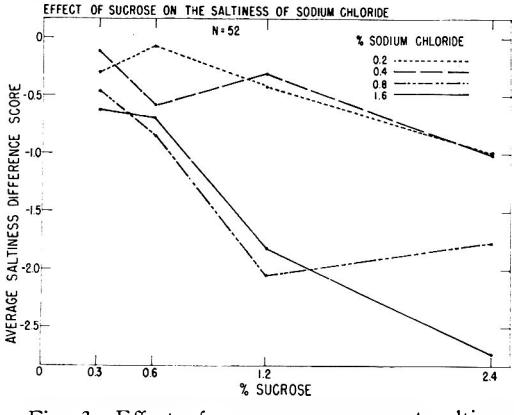

Fig. 3. Effect of sucrose on apparent saltiness of sodium chloride in canned tomato juice.

Study IV. Effect of sodium chloride on apparent sweetness of sucrose. Sodium chloride at concentrations of 0.05, 0.1, 0.2, 0.4, and 0.8% significantly enhanced the sweetness of all levels of sucrose (Table 1, Fig. 4). At 1.6% sodium chloride, however, apparent sweetness was significantly depressed. The analysis of variance shows no variation due to sucrose levels or replications, but very large variation due to salt levels and judges. Divergence of opinion between judges and between groups is readily seen in Table 5, where only Group II was in complete agreement and assigned the highest positive average difference scores.

Study V. Effect of citric acid on apparent saltiness of sodium chloride. This study was the

Table 3. Analysis of variance of difference scores. Interaction of sucrose and sodium chloride.

Source of variation	III. Sucrose-saltiness [a]		IV. Sodium chloride-sweetness [b]	
	Degrees of freedom	Mean square	Degrees of freedom	Mean square
(S) Sucrose	3	70.02***	3	3.46
(N) NaCl	3	55.57***	5	315.19***
(R) Replications	3	1.72	3	2.50
(J) Judges	12	12.97***	12	232.76***
(G) Groups	2	2.13	2	318.60***
(Y) Judges within groups	10	15.14***	10	215.59***
S × N	9	9.57**	15	4.49**
S × R	9	0.65	9	4.08*
S × J	36	5.28*	36	4.74***
G × S	6	6.91	6	5.38**
Y × S	30	4.95	30	4.61***
N × R	9	1.45	15	3.46*
N × J	36	6.54**	60	27.57***
G × N	6	3.79	10	25.87***
Y × N	30	7.09**	50	27.91***
R × J	36	3.90	36	1.81
G × R	6	1.20	6	1.20
Y × R	30	4.44	30	1.93
S × N × R	27	4.64	45	2.04
S × N × J	108	5.26**	180	3.56***
S × R × J	108	3.10	108	1.94
N × R × J	108	2.33	180	2.23*
Remainder [c]	324	3.32	540	1.81

[a] Effect of sucrose on saltiness of sodium chloride.
[b] Effect of sodium chloride on sweetness of sucrose.
[c] Error term (S×N×R×J).
*, **, *** Respectively significant at p = 0.05, 0.01, and 0.001.

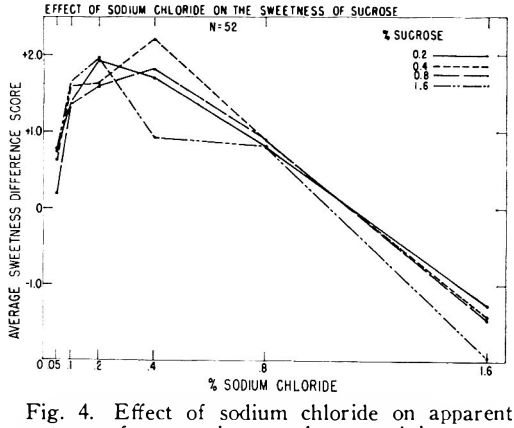

Fig. 4. Effect of sodium chloride on apparent sweetness of sucrose in canned tomato juice.

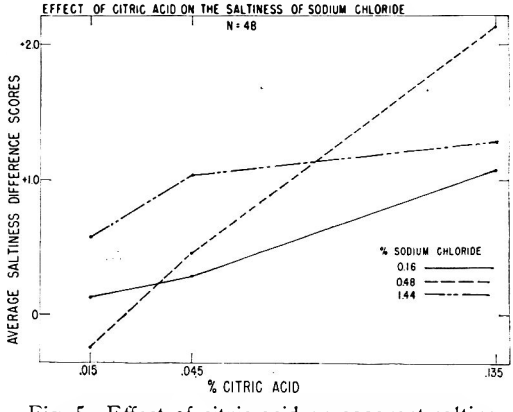

Fig. 5. Effect of citric acid on apparent saltiness of sodium chloride in canned tomato juice.

final one of the series, and, because of exhaustion of the tomato juice supply, it was necessary to use fewer concentrations. Note, however, that the range of concentrations selected for both the salt and the acid approximate the ranges used in the previous studies. Table 1 and Fig. 5 show that the acid enhanced the saltiness of the three levels of sodium chloride. Judges differed from each other slightly, but groups agreed on the pattern of general enhancement of apparent saltiness.

Study VI. Effect of sodium chloride on apparent sourness of citric acid. A picture of general depression of sourness by the addition of 0.02 to 0.32% sodium chloride is presented in Table 1

and in Fig. 6. All citric acid levels were affected similarly. Judges generally agreed on direction of response, except for a high positive value assigned by Judge 8 (Table 5). Again, replications and J × R were not significant (Table 4).

Influence of original sensitivity on reliability of response. As indicated previously, although all thirteen judges fell within the established range of acceptability on the basis of their sensitivity to sweetness, sourness, and saltiness in tomato juice, there were three sublevels of sensitivity among the thirteen judges. It was immediately apparent from the statistical analysis that these groups gave significantly different responses in all but two studies. Since the method requested an indication of apparent taste intensity, there were no "right" or "wrong" responses. Consequently the data were grouped according to the reproducibility of opinion of the three groups when judging duplicate pairs. In all six studies the frequency of reversal of direction of response within a set was higher for judges of the lower sensitivity. For the low-, medium-, and high-sensitivity subgroups, respectively testing 1680, 1564, and 2100 duplicate pairs, the percentage of reversals was 15.8, 15.2, and 8.8. Chi-square analysis showed that these percentages differed significately at p = 0.001.

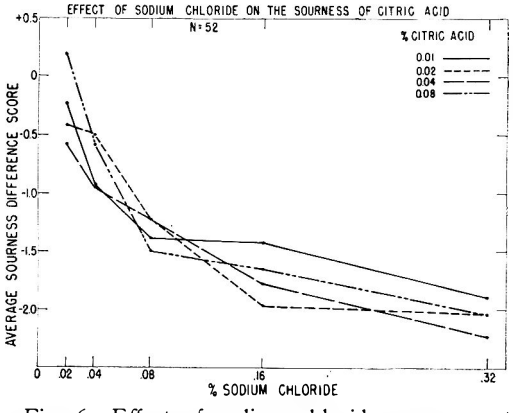

Fig. 6. Effect of sodium chloride on apparent sourness of citric acid in canned tomato juice.

DISCUSSION

Although different judges participated, the average taste intensity scores reported for

Table 4. Analysis of variance of difference scores. Interaction of citric acid and sodium chloride.

Source of variation	V. Citric-saltiness [a]		VI. Sodium chloride-sourness [b]	
	Degrees of freedom	Mean square	Degrees of freedom	Mean square
(C) Citric acid	2	63.45***	3	2.38
(N) NaCl	2	6.86	4	108.84***
(R) Replications	3	1.96	3	1.40
(J) Judges	11	16.67***	12	109.38***
(G) Groups	2	1.30	2	132.44***
(Y) Judges within groups	9	20.09***	10	104.77***
C × N	4	12.51***	12	2.59
C × R	6	2.47	9	2.16
C × J	22	6.42***	36	4.12*
G × C	4	1.79	6	6.08*
Y × C	18	7.46***	30	3.73
N × R	6	6.18*	12	1.79
N × J	22	4.57**	48	12.40***
G × N	4	4.97	8	9.69***
Y × N	18	4.48*	40	12.94***
R × J	33	4.06*	36	2.85
G × R	6	3.48	6	3.03
Y × R	27	4.19*	30	2.81
C × N × R	12	1.82	36	2.25
C × N × J	44	2.98	144	2.45
C × R × J	66	3.27*	108	2.96
N × R × J	66	2.92	144	2.37
Remainder [c]	132	2.28	432	2.86

[a] Effect of citric acid on saltiness of sodium chloride.
[b] Effect of sodium chloride on sourness of citric acid.
[c] Error term (N×R×C×J).
*, **, *** Respectively significant at p = 0.05, 0.01, and 0.001.

Table 5. Average difference scores assigned by individual judges.[a]

Group[b]	Judge	Sex		I Sucrose-sourness	II Citric-sweetness	III Sucrose-saltiness	IV NaCl-sweetness	V Citric-saltiness	VI NaCl-sourness
			n	64	80	64	96	36	80
High	1	M		−0.94	−1.25	−1.44	−0.65	+0.78	−1.58
	2	F		−1.25	−0.85	−1.00	+2.06	+1.50	−1.58
	3	F		−1.19	−2.15	−1.59	−2.29	+0.67	−2.55
	4	F		−2.19	−1.43	−0.72	+0.33	−0.28	−1.98
	5	F		−1.72	−0.23	+0.03	+2.38	+0.56	−1.55
			$\bar{x}$	−1.46	−1.18	−0.94	+0.37	+0.64	−1.85
Medium	6	M		−0.50	−0.55	−0.66	+2.00	+0.78	−1.15
	7	M		+0.06	−1.43	−0.81	+1.92	+2.00	−2.45
	8	M		−0.41	....	−0.50	+1.04	....	+1.50
	9	M		−1.59	−1.78	−1.34	+2.54	−0.39	−1.55
			$\bar{x}$	−0.61	−1.25	−0.83	+1.88	+0.80	−0.91
Low	10	M		−0.66	−0.68	−0.63	+2.19	+1.33	−2.30
	11	M		−1.06	−0.83	−1.34	+0.58	+0.83	−0.55
	12	M		+0.16	−0.63	−1.16	−0.56	+0.78	+0.30
	13	F		−0.38	−0.50	−0.91	−1.08	+0.28	−0.35
			$\bar{x}$	−0.49	−0.66	−1.01	+0.28	+0.81	−0.73
Difference[c]				2.35	1.92	1.62	3.62	2.39	4.05

[a] Negative scores indicate the compound depressed apparent taste intensity. Positive scores indicate the compound enhanced apparent taste intensity.
[b] Grouped on the basis of sensitivity to sweetness, sourness and saltiness determined by triangular testing during original training period.
[c] Difference between highest and lowest value within each study.

the tomato juice are in excellent agreement with those for water solutions (Pangborn, 1961; 1962) and for lima bean purée (Pangborn and Trabue, 1964a). Individual judge variability was substantial in all studies, yet, in all media, sucrose and citric acid exhibited mutual masking effects, as did sucrose and sodium chloride, except for a slight enhancement of sweetness by low levels of sodium chloride. Citric acid enhanced all levels of sodium chloride whereas sodium chloride depressed the sourness of citric acid. The latter relationship was more clear-cut in the tomato juice than in the purée samples. There was no prior assurance that there would be agreement between the two media, for lima bean purée is a thick, starchy, bland product in which sweet and sour compounds occur at or below threshold levels, and saltiness is slight. Commercial tomato juice, in contrast, contains between 0.66 and 1.1% sodium chloride (Anderson *et al.*, 1954) and its sourness and sweetness are readily perceptible. Another variation was that of temperature of serving—165°F for the purée and 70°F for the tomato juice. To determine the influence of temperature on taste responses, it would be necessary to standardize the amount of solution ingested and the length of time it remains in the mouth. Although the rate with which the liquid passes over the tongue may be controlled, the temperature of the receptors may be of more importance than that of the stimulating substance.

It is generally recognized that experimental judges differ substantially in sensitivity and in reproducibility of sensory response, yet most investigators in food psychophysics ignore this source of variation by reporting mean values, only. In many cases, the variation is reported as the error term in the analysis of variance. Early in our studies on the interaction of the basic tastes we noted variation among judges in ability to detect differences, and in rating of taste

intensities; some judges consistently used the upper, and others the lower, part of the rating scale. Furthermore, with the experimental method used herein, the judge evaluates "apparent" sweetness, sourness, or saltiness. There is no right or wrong response, but between- and among-judge variability could occur as a result of shifting criteria of interpretation of these sensations in the presence of a secondary compound. In informal conversations with some judges, we noted a stated intention to achieve consistency of response. Some subjects decided during the training period that one compound depressed or enhanced apparent taste intensity, then attempted to maintain this direction of response throughout the study. As indicated by Jones and Marcus (1961), individual differences in judgment of taste stimuli may be ascribed to perceptual differences or to differences in habits of response.

In the present study, some judges remarked that the influence of the added compound altered the character of the secondary compound rather than the apparent intensity. If an appropriate method could be developed, a modification of the so-called time-intensity study (Neilson, 1958) could be used to establish the temporal and sequential changes in apparent taste intensity of both compounds in a mixture. The procedure would present some difficulties since the oral stimulation is relatively rapid.

Gregson and McCowen (1963) recently observed that some judges generally considered citric acid to increase sweetness in water solutions whereas other judges reported a decrease. Those investigators agree that it is misleading to treat all tasters as equivalents, and that judge effects should be separated in the analysis of the data.

In the present study, judges were selected on the basis of their ability to discriminate, then further subdivided into three levels of sensitivity. Results do not indicate that this original sensitivity influenced the direction of the response, i.e. whether the secondary compound enhanced or depressed the apparent intensity of the primary, but it was manifested somewhat in the magnitude of the difference scores (Table 5) and in the reproducibility of judgment. In retrospect, it would have been of interest to have retested the judges' discriminability at the termination of the study to determine whether the experience of the extended tasting influenced sensitivity.

Current investigations are showing that there is little relationship between the ability to detect a taste in the presence of additional compounds and the effect of one compound on the apparent intensity of another (Pangborn and Ough, 1964; Pangborn and Trabue, 1964b). Once again, even these relationships are influenced by individual judge variability.

ACKNOWLEDGMENTS

The interest and cooperation of the members of the panel is sincerely appreciated. The assistance of Dr. P. W. M. John, Department of Mathematics, and of Mrs. Ida M. Trabue, I. D. Desai, William Wildeman, and Frank Winter is gratefully acknowledged. The investigation was supported, in part, by the Sugar Research Foundation, Inc., New York.

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Linear Dependency of Scale Structure in Differential Odor Intensity Measurements

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(Manuscript received August 31, 1963)

SUMMARY

Data are presented showing that the subjects' basic reaction to situations where scaling of odor differences are concerned, is basically the same as far as their scale structure is concerned. The relative differences between scale-point means are essentially the same. The major differences are the degrees to which they use the scale range, the location of the central value, and their discriminating ability in a few instances. Some scale positions are much more stable than are others.

A study was made of paired-comparison and intensity rating methodology, and it was concluded that intensity rating gave equally as much information as the paired comparison method in a shorter time.

INTRODUCTION

The differences between subjects' responses to various sensory situations have been a thorn in the side of investigators. Baker *et al.* (1958), Mrak *et al.* (1959), Ough (1963), and Amerine *et al.* (1962) have shown that differences between subjects' responses to various test situations are persistent and real. Nelson *et al.* (1963) found that the use of hedonic scales varied considerably between subjects. The obvious cause of concern over this variability is the difficulty in statistical analyses. Some may combine the data and use techniques that ignore this variability, but techniques must be developed to handle these variations or much of the sensitivity of strong statistical methods is lost.

This investigation studied eleven subjects' responses to 2-heptanone using two methods of presentation for determination of differential odor intensities in an effort to relate the subjects' responses to the methodologies and to study the structure of the responses.

METHOD

The equipment used, described by Ough and Stone (1961), is designed to deliver to a subject a definite odor intensity, free from extraneous odors, that can be varied easily and is reproducible. The test material was chosen because of its proven use over a three-year period as a stable, relatively nonfatiguing compound.

Two methods of presentation were used. A standard modified constant-stimulus paired comparison technique described in detail by Stone *et al.* (1962) and an intensity-rating method. The second method entailed presenting the subjects with a reference odor level and then giving them 14 odor levels in succession and asking them to rate the odor intensity on a word intensity scale in comparison to the reference. The subjects were instructed to consider the reference as the *moderate,* and that they could receive (and did) the reference again in the test levels. The ratings were as follows: Very very weak, very weak, weak, moderate, strong, very strong, and very very strong. When the data were tabulated the intensity-ratings were assigned scale values of 1 through 7 (-3, -2, -1, 0, $+1$, $+2$, $+3$) for purposes of computation.

The same test levels of 2-heptanone were used for both methods of response: 0.54, 0.72, 0.81, 0.90, 0.99, 1.08, and 1.26 mg/min of test material metered into a steady mainstream air flow of 13 cu ft/min. The reference level was 0.90, and the differences were coded -4, -2, -1, 0, $+1$, $+2$, and $+4$ for ease of handling. In both experiments the time intervals for the odor levels was odor for 10 seconds and then no odor for 10 seconds.

The order of presentation within sets was made by selecting random numbers from one to twelve from a table of random numbers. The within-pair order was determined by referring to the random-number tables and assigning the pair order accord-

ing to whether the number from the table was odd or even. Separate orders were made for each subject to the extent of giving them 10 sets; each set contained two complete duplicates. For the intensity-rating method the same orders were used for the test levels, and two of the reference levels were selected, again by reference to the random-number table. The subjects were assigned one set per day for the paired response, and a different order was set for the intensity-rating response.

The subjects were given one day of training but given only the necessary information about the test. No information was available to them on differences in intensity or other matters.

Subjects were given the series over a 10-day period. On alternate days they received the paired-comparison series first, and the intensity-rating series second. This was reversed on the other days. For the paired-comparison a practice pair was given initially; then the 12 test pairs were given. The air lines and hood were saturated for several minutes with the reference test level before subjects entered the hood.

A wide spectrum of subjects was used for the tests. Table 1 indicates individual experience and training. Age of the panel varied from middle twenties to early fifties. Subject five was the only female. All subjects were staff or students in the Department of Viticulture and Enology. Subjects 1, 3, 4, and 11 could be considered highly trained both in the use of the apparatus and in familiarity with 2-heptanone.

Probit, variance, regression, and factor analyses of the data were done as indicated by Baker (1962) and Cattell (1952). Regression lines were estimated for the paired-comparison data as indicated by Jones (1957).

RESULTS AND DISCUSSION

Table 2 lists the intensity-rating data by subjects by presentation order (whether presented before or after the paired test) and by mean scale value. The combined data were subjected to analysis of variance (Table 3), which indicated that all main order effects were significant as well as both subject interaction terms. The mean difference between the order of presentation was 0.338 and was larger for the second set presentation. However, the order of presentation × level interaction was insignificant, indicating that the responses were similar but were displaced along the scale continuum. This was verified by a plot of the data. This is believed to be a first-sample effect. When the subjects were given the reference sample as the first sample it seemed relatively more intense than the following samples or when the intensity-rating set was given after the paired test. The analysis of variance was further partitioned to show the effects of the regression and the deviations from regression (Davies, 1956). The error term used is the pooled value for the partitioned three-way interaction and a fixed effects model is assumed (see Scheffé 1959).

Regression analysis of the combined data (Table 2) is shown in Table 4. Real differences exist in the slope values between subjects; this can also be seen in Table 3 with the levels × subject interactions.

Table 1. Experience and training of individual subjects.

Subjects	General[a] experience (years)	Experience with olfactometer	
		Type of tests	Years
1	4	Difference and threshold	3[b]
2	20	Difference and threshold	1[c]
3	6	Difference and threshold	3[b]
4	10	Difference and threshold	3[b]
5	.....	.....	.....
6	.....	Difference	8 hr[d]
7	.....	.....	.....
8	4	Difference and threshold	1[c]
9	½	.....	.....
10	½	.....	.....
11	15	Difference and threshold	3[b]

[a] "General" experience indicates service on tasting and odor panels on a daily or twice-daily basis for 6 to 9 months of the year.
[b] Testing to date using 2-heptanone two hours a week continuously.
[c] Testing using 2-heptanone two hours per week two years previous to these experiments.
[d] Testing using other than 2-heptanone and done 18 months prior to these experiments.

Table 2. Intensity-rating data—mean scale values.

Presentation order	Difference level	Subjects										
		1	2	3	4	5	6	7	8	9	10	11
First Set	−4	1.4	1.5	1.4	1.5	2.1	1.7	1.8	1.8	1.1	1.0	1.1
	−2	1.8	2.1	2.1	2.5	2.9	2.6	2.6	2.3	2.0	1.5	1.5
	−1	2.4	2.9	2.7	3.2	3.1	3.5	3.1	3,9	3.3	2.2	2.3
	0	3.3	3.7	3.5	3.6	3.2	4.2	3.5	3.4	3.7	2.4	2.8
	1	4.2	4.3	4.0	4.1	4.8	4.5	4.0	3.9	4.5	2.9	3.4
	2	4.8	4.7	4.5	5.0	5.5	3.9	4.2	4.1	5.4	3.1	4.2
	4	5.6	6.1	6.7	5.4	6.7	5.3	5.2	5.1	5.8	4.5	4.7
Second Set	−4	1.4	1.4	1.4	1.4	1.8	1.7	2.2	2.6	1.3	1.2	1.0
	−2	1.9	2.3	2.6	2.6	2.5	2.3	2.4	2.7	2.4	3.0	1.7
	−1	3.2	3.6	3.1	4.3	4.5	2.9	2.9	3.8	3.7	2.8	2.4
	0	3.2	4.1	3.9	4.0	3.3	3.5	3.1	3.7	4.8	3.4	3.0
	1	4.7	4.4	4.9	5.0	5.8	3.7	3.7	4.2	5.0	4.8	3.4
	2	5.4	4.7	5.1	5.3	4.7	4.5	4.8	4.8	5.7	5.1	4.5
	4	6.8	5.9	6.6	6.3	6.3	5.2	5.3	5.8	6.5	6.2	5.2
Combined	−4	2.8	2.9	2.8	2.9	3.9	3.4	4.0	4.4	2.4	2.2	2.1
	−2	3.7	4.4	4.7	5.1	5.4	4.9	5.0	5.0	4.4	4.5	3.2
	−1	5.6	6.5	5.8	7.5	7.6	6.4	6.0	7.7	7.0	5.0	4.7
	0	6.5	7.8	7.4	7.6	6.5	7.7	6.6	7.1	8.5	5.8	5.8
	1	8.9	8.7	8.9	9.1	10.6	8.2	7.7	8.1	9.5	7.7	6.8
	2	10.2	9.4	9.6	10.3	10.2	8.4	9.0	8.9	11.1	8.2	8.7
	4	12.4	12.0	13.3	11.7	13.0	10.5	10.5	10.9	12.3	10.7	9.9

Table 3. Intensity-rating data—analysis of variance.

	df	s.s	m.s	F	$F_{.05}$	$F_{.01}$
Order	1	4.39	4.39	41.03	4.00	7.08
Subjects	10	15.72	1.572	14.69	1.99	2.63
Levels R^1	1	276.44	276.44	2,583.55	4.00	7.08
Levels D^2	5	2.27	.454	4.24	2.37	3.34
OXL R^1	1	.51	.51	4.77	4.00	7.08
OXL D^2	5	.23	.056	.52	2.37	3.34
OXS	10	5.49	.549	5.13	1.99	2.63
LXS R^1	10	7.04	.704	6.58	1.99	2.63
LXS D^2	50	7.17	.143	1.34	1.56	1.87
OXLXS R^1	10	.32	.032	.26	2.02	2.70
OXLXS D^2	50	6.10	.122			

[1] Regression.
[2] Deviation from regression.

Some subjects used a narrow portion of the scale, whereas others used broader sections. The scales used seem to be individual but can be transformed into each other by a linear transformation. This means that the origins of the scales may be shifted and that the units of measurement for the scales may be changed. An 11 × 11 correlation matrix was calculated for the intensity-rating data. Correlations within the matrix were extremely high, from 0.93 to 1.00, and the F-values computed ranged from 0.97 to 1.00, indicating the subjects' scaling to be linearly dependent. This finding is further supported by Ough *et al.* (1964). The factor due to difference in concentration, accounts for all of the structure in the correlation matrix (see Cattell, 1952).

Table 4. Intensity-rating data—regression analysis.

Subjects	Intercept a	Slope b	Standard error of estimate S_e	Standard deviation of slope S_b
1	3.58	.65	.34	.052
2	3.70	.58	.20	.031
3	3.76	.66	.23	.035
4	3.87	.56	.28	.043
5	4.09	.58	.51	.079
6	3.54	.44	.21	.032
7	3.49	.43	.17	.026
8	3.72	.41	.30	.046
9	3.94	.66	.35	.054
10	3.15	.53	.18	.028
11	2.94	.53	.23	.035

Table 6. Paired-comparison data-responses [a] called stronger than reference.

Subject	Coded concentration differences					
	−4	−2	−1	1	2	4
1	0	3	5	13	17	20
2	2	5	9	13	18	18
3	1	6	3	17	20	20
4	0	2	5	13	14	19
5	1	6	10	10	18	19
6	1	3	9	11	18	20
7	4	4	6	11	19	19
8	2	4	8	17	19	20
9	0	2	5	14	19	19
10	3	3	5	14	17	19
11	1	1	6	15	17	20

[a] 20 replicates at each point for each subject.

Table 5 shows that certain concentrations were rather consistently rated by all subjects whereas other concentrations were very unstable in rating. This finding is also supported by Ough *et al.* (1964). Large values of F indicate high stability, and low F-values indicate low stability.

Responses stronger than the reference are tabulated in Table 6 for the paired-comparison constant-stimulus data. Table 7 shows the values for probit analysis of the data. The combined standard error of estimate for the differences between the regression coefficients is approximated at 2.32. With these data it was also possible to show a high correlation between subjects' scaling. The correlation matrix calculated had correlation coefficients from 0.86 to 1.00 and F_1 values from 0.95 to 1.00. Perhaps "scaling" is an improper term since the subjects could not scale more than (+1, −1). However, the percentage called more intense may be treated as a scale in effect since it shows progressive change over the range. The correlation matrix corresponding to Table 6 is given in Table 8 and shows that instability among subjects was greater for paired-

Table 7. Paired-comparison data—probit regression line values.

Subject	Intercept a	Regression Coefficient b	Standard error of estimate of slope S_b
1	5.05	13.13	1.86
2	5.13	7.29	1.25
3	5.21	15.05	1.71
4	5.02	11.82	1.89
5	5.15	7.93	1.43
6	5.11	10.17	1.47
7	5.04	6.62	1.36
8	5.21	10.30	1.39
9	5.12	13.50	2.24
10	5.04	10.56	1.47
11	5.10	11.65	1.96

Table 5. Combined intensity-rating data—correlations between subjects for different scale values and factor weightings for the first factor.

Odor intensity	1	2	3	4	5	6	7
1	(.80) [a]	.70	.64	.09	.21	−.07	.01
2	.70	(.80)	.76	.33	.52	.13	.29
3	.64	.76	(.80)	.56	.69	.42	.38
4	.09	.33	.56	(.60)	.48	.47	.31
5	.21	.52	.69	.48	(.90)	.79	.86
6	−.07	.13	.42	.47	.79	(.80)	.74
7	.01	.29	.38	.31	.86	.74	(.90)
Total	2.38	3.53	4.25	2.84	4.45	3.28	3.49
F_1	.483	.717	.863	.576	.903	.665	.708

[a] The figures in parentheses are assigned according to Cattell (1952).

Table 8. Factor analysis on paired-comparison data—correlations between tasters.

Odor intensity	1	2	3	4	5	6
1	(.35)	.28	.15	.09	.33	−.27
2	.28	(.60)	.29	.07	.54	.19
3	.15	.29	(.60)	.52	.06	.25
4	.09	.07	.52	(.70)	−.24	.67
5	.33	.54	.06	−.24	(.60)	−.17
6	−.27	.19	.25	.67	−.17	(.70)
Total	.93	1.97	1.87	1.81	1.12	1.37
F_1	.309	.654	.621	.601	.372	.455

comparison data than for intensity-rating data.

What, in essence, is shown with the scaling data from both presentations, is that the individual scales of the subjects are all linearly related and that the subjects all react to give scales of the same structure. Some subjects are more discriminating than others and are capable of giving more information with fewer tests, but the way each scales the data is very similar. It is also noted that some relative scales positions are rated much more consistently than others.

Helson *et al.* (1954) were the first to introduce the intensity-rating scale for the evaluation of psychophysical data. They used a nine-point intensity scale for judgment of five weights and showed plots of log weights against linear scale assignments to yield straight lines. Very little difference in the dispersion was noted over this scale. With the combined intensity-rating scale data of this report the best fit was to linear scale and linear concentration differences.

The slope values for the two methods and the subjective ranking of the subjects are compared in Table 9. Previous data on many of the subjects were insufficient for estimation of a good subjective rank to be assigned; however, the assigning was done independently of knowledge of the final results of the experiments, and the correlations do show the subjective rankings to be in the right direction. The subjective rank was obtained by review of the subjects' previous records and arbitrarily assigning the ranks on the relative size of error terms or number of correct paired testing decisions. The correlation of the slope value between the methods is somewhat better and, if two of the

Table 9. Correlation of slope values for intensity-rating and paired comparisons, and a subjective ranking of subjects.

Subject	Subjective rankings (A)	Slope values: Intensity-rating (B)	Slope values: Paired-comparison (C)
3	1	0.66	15.05
2	2	0.58	7.29
8	3	0.41	10.30
1	4	0.65	13.13
9	5	0.66	13.50
11	6	0.53	11.65
10	7	0.53	10.56
4	8	0.56	11.82
5	9	0.58	7.93
6	10	0.44	10.17
7	11	0.43	6.62

Comparison	Correlation coefficient
A vs. B	−0.500
A vs. C	−0.489
B vs. C	0.593
B vs. C (− subjects 2 and 5)	0.865

subjects are discarded, becomes quite good. It is shown that the subjects, in general, reacted with approximately the same relative efficiency to both tests. The intensity-rating seems to be somewhat more stable and consistent than for paired comparisons.

The variance of the data from the replicate mean values for the intensity-rating was calculated (Table 10). Subject variability was quite high, and the subjects could be divided into several homogeneous groups. The variances associated with the individual scale means show a tendency to be smaller at the extremes, and these changes are roughly proportional to the concentration change. Figs. 1 and 2 plot the mean data for the intensity-rating and the paired-comparison. Both give linear regression lines. The regression line for comparison-rating data was calculated by the method of least squares, and that of the paired comparison by the maximum likelihood methods as outlined by Jones (1957). The data in both instances indicated very good fit, as may be seen by the standard errors of estimates for y and B in the figures. The combined error of a predicted y from a value of x less than ± 0.09 from the reference and of the y of the reference x was calculated for each method from the calculated regression lines [(S_y^2 =

Table 10. Variances of the deviations about the individual replicate means.

	Subjects											
df	1	2	3	4	5	6	7	8	9	10	11	Total
126	1.084	0.973	1.638	1.138	1.849	1.030	1.849	0.605	1.642	0.959	0.998	1.251

		Odor differences level (coded)								
df	Presentation of set order	−4	−2	−1	0	+1	+2	+4	Sub-totals	Total
99	1st	0.463	1.360	1.333	1.831	1.751	1.337	1.178	1.320	
99	2nd	0.515	1.020	1.565	1.935	0.957	1.422	0.869	1.183	
	Sub-totals	0.488	1.190	1.449	1.883	1.354	1.372	1.024		1.251

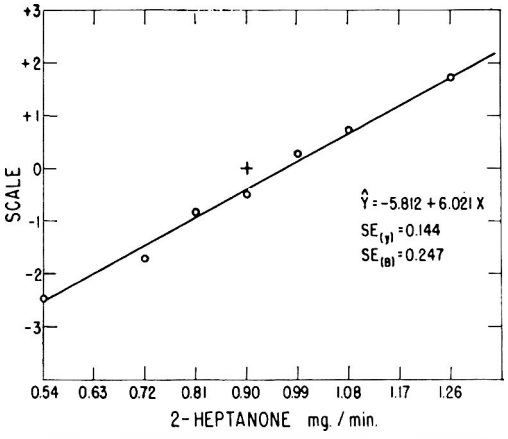

Fig. 1. Combined intensity-rating data—mean values of the scale rating versus the concentration of 2-heptanone. Line of best fit calculated by method of least squares.

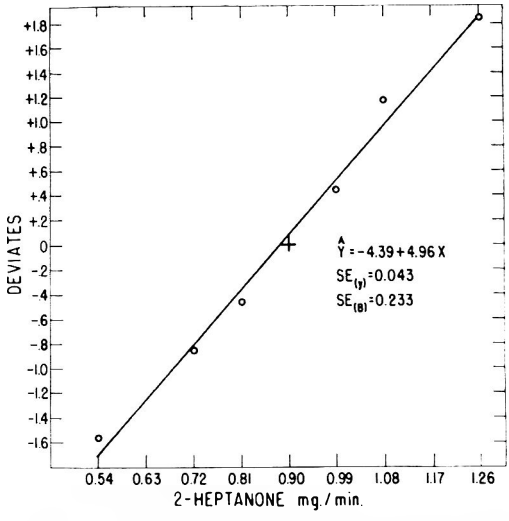

Fig. 2. Combined paired-comparison data—mean values as normal deviates of percent answers called stronger than the reference value versus concentration of 2-heptanone. Line of best fit calculated by the method of maximum likelihood.

$Sy \cdot x^2 + Sy \cdot x^2 n + Sy \cdot x^2 X^2 / SX^2$) see Baker (1962)]. These values times 1.96 (t .05, 200 df) give the difference of y that is significantly different. This difference in y is proportional to a difference of x and related to the slope value. Dividing by the slope gives the approximate difference in x for the subjects to be able to discriminate between levels (± from the reference). Using the appropriate x values, any points along the line may be tested for significant difference. These data, and also the least significant difference calculated from estimate of the error term as approximated by the variance from the individual means for the intensity-rating data and the 50% above chance difference thresholds, are given in Table 11. Using these approximations of the variance about the individual means is generally a good estimate of the reduced error term as calculated in the analysis of variance. This term will not include subject interactions, and would probably be a low estimate of s_r.

The most discriminating group estimation of the least significant difference of 2-heptanone that was obtained by the methods used was that calculated from the regression data of the intensity-rating experiment. A slightly lower difference threshold was obtained with the paired-comparison method. The differences based on the regression analysis are not significant.

The intensity-rating data treated as paired-comparison data, using the signs of the values as indication of more intense odor than the reference and dividing "0" equally between more and less intense calls, calculated by the method of Jones (1957), gave almost identical results to that of the paired-comparison data—$\hat{y} = -4.74 + 5.01x$; $SE(y)$

Table 11. Comparison of the relative amounts of 2-heptanone for significant difference from the reference level and the difference thresholds by the two methods.

	Regression analysis		Analysis of variance
	Paired-comparison	Intensity-rating	Intensity-rating
Combined standard error of y from x [a]	0.206	0.217	$S_{\bar{x}}$ [b] 0.075
Slope values	4.96	6.02	
5% level of significance of x from references	±0.081 mg/min	±0.071 mg/min	LSD 0.208 mg/min
50% above chance difference threshold	±0.136 mg/min	±0.146 mg/min [c]	

[a] Based on the differences from the regression line $\left(Sy^2 \cdot x = \sum \frac{(y-\hat{y})^2}{n-2} \right)$ in both cases. Calculated to give a combined standard error of the values of y predicted from x within a range of ±0.09 of x from the reference value.

[b] Calculated $s_{\bar{x}}$ as $\sqrt{1.251/200}$ from Table 10.

[c] Calculated from regression equation based on the signs of the intensity-ratings.

= 0.039; and $SE(B)$ = 0.233. The only major difference was the intercept value, and this difference was due to the first-sample effect, discussed earlier.

The intensity-rating gives results that are at least equivalent to those obtained by paired-comparison techniques. The time used per subject per day by intensity-rating was 4.8 minutes and for the paired-comparison 8.5 minutes, the paired-comparison method taking about 1.8 times as long.

ACKNOWLEDGMENTS

Deep appreciation is expressed to the referee whose considerate and helpful suggestions greatly improved the treatment of the data. The sound advice of Professor Peter W. M. John, Department of Mathematics, University of California, Davis, is acknowledged, as well as the computing services of Mr. Anthony M. Fratis.

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A Comparison of Normal and Stressed-Time Conditions on Scoring of Quality and Quantity Attributes

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(Manuscript received August 31, 1963)

SUMMARY

Data reported indicate that tasting under a time stress may be slightly advantageous when subjects are asked to judge a quantity difference; but that for judging quality differences, tasting with no time stress is superior for most tasters. The Brunswick probabilistic theory is discussed in the light of these experiments, which tend to verify applicability of the theory. The linear dependency of tasters' use of scales for quantity evaluation, and to some extent for quality evaluation (if not under stress), is demonstrated.

INTRODUCTION

Taste processes and individual responses to various quantity and quality attributes need further investigation. Brunswick (1956) has offered a theory, in the field of psychology, that there are two general attitudes in perception. One attitude he has called "perceptual." This can be considered as a quick statistical mind-average. The other is "intellectual" and implies the use of evaluation, meditation on the individual attributes, and calculation with critical thought. In his demonstration experiments, the techniques and conditions were such as to allow large response differences. For example, if difference in the height of two bars was judged by a group allowed only a brief glance at the comparison, a normal distribution of scores about the true mean was demonstrated. However, if ample time was allowed for thought and contemplation and the test conditions were such that there were several possible but only one true answer the distribution of scores tended to be bi- or multi-modal and was influenced to a greater extent by the conditions of the test. The distributions with "intellectual" judgments were very narrow and centered on each of several means, and were based on judgments that were of a calculated type rather than a quick perceptual judgment. Calculated judgments were shown to yield several responses, of which only one was the correct answer.

Tasting of wines, or any food or beverage, is based on the perceptive ability of the subject and on the material tasted as well as the conditions during tasting. In quality judgments one would expect an "intellectual" type of judgment to be used. Experience would be essential, and the various factors involved in quality of the particular product must be weighed and evaluated critically. "Perceptual"-type judgments would seem more appropriate for quantity-type judgments.

The experiments reported here were done to determine whether judges would respond differently when time stress was imposed during tasting than they would under normal (non-time stress) tasting conditions. The idea of the time stress was to force the subjects to use "perceptual" responses. Both quality and quantity attributes were tested.

METHOD

The panel used was 10 male judges, 25 to 55 years old. They had had 3–25 years of wine-tasting experience, and all are considered reasonably or expertly proficient at this task.

Quantity judgments were asked of the subjects on a series of 10 wines ranging from 0.0 to 3.6 g of added sucrose per 100 ml of wine. Wines were

made up daily by adding sucrose to ½ of the required sample and diluting the sweetened sample with unsweetened wine to give 10 levels of sucrose with a difference of 0.4 g/100 ml between levels. These different levels of sugar in wines were numbered 1 to 10 with increasing sugar concentration, for ease of enumeration in the tables. The base wine was a standard white wine of commercial quality containing only about 0.1 g of reducing sugar per 100 ml, and having no detectable sweetness (Hinreiner *et al.*, 1955).

For the quality judgments, two red sweet wines were blended to give a series of 10 wines ranging from "poor" to "good" in quality. Blends were numbered 1 to 10 with increasing quality. These two wines had been tasted several times by previous panels, and out of a prospective group of wines had been categorized as "poor" and "good." The "poor" port was made from late-harvested underripe Zinfandel grapes, and the "good" port from a blend of ports made from Royalty and Tinta Madeira grapes in excellent condition. Analytical data concerning these wines are given in Table 1. Color differences existed between these two wines. To minimize the use of color alone as a basis of differentiation, the wine was served in black glasses under red lighting, and black spittoons were used.

The tasting was done in an air-conditioned room with standard booths. The glasses were coded with two-digit numbers in excess of the integer 20. Ten randomized 10×10 Latin squares were used to determine order of presentation. Each square was split into two equal sections. Each taster was assigned ½ of one square, and ½ of another. One half square was assigned for the normal time condition, and the other for the stress time. The five samples for each time condition were given each day. One day the time stress was first; the next the normal condition was first. The subjects received no daily duplicates within each time condition, but may or may not have received them between the two time conditions. At the conclusion of the use of the orders, the blocks were assigned opposite time conditions and the experiment repeated. This should have eliminated any order effects on the time conditions. Five days' training was given before the sugar level experiment was begun. At the conclusion of the quantity experiment, the half blocks were reassigned to different subjects and the quality experiment started with a two-day practice session.

The subjects were given two reference samples corresponding to the two end samples in each series (zero and 3.6 g sugar added in the sweetness or quantity series, and 100% "poor" and 100% "good" wine in the quality series) prior to their tasting of each of the five daily samples under each time condition. The reference samples were removed prior to each series of 5 tests. They were asked to score the test samples from 1 through 10, the higher score referring to the larger sugar concentration in the quantity experiment—and the better quality in the quality experiment.

Under the stressed-time condition the subjects were allowed 9 seconds to pick up the wine glass, smell (if needed) and taste, expectorate, and report the assigned score orally. A rest period of 30 seconds was allowed between samples. Under the normal time conditions, no limit was set on their tasting speed or their rest time between samples, and they were allowed to record their score with pencil and paper at leisure. The stressed-time condition was established by trials made prior to the start of the experiments. Trout and Sharp (1937) showed that sample time, in the mouth, for simple quantity factors averaged 5–10 sec and that a judge could rank a series of ten samples in 3–6 min. Helm and Trolle (1946) allowed their subject 15 min to evaluate a triangle set of beer for quality. Based on these facts and the reactions of the judges in the practice sessions, the 9-second time limit was considered a real stress condition even for the quantity evaluation.

RESULTS AND DISCUSSION

Tables 2 and 3 show the results of the mean scores (10 replicates) for the 10 tasters under the two test conditions for both quantity and quality experiments. The most noticeable difference is the higher scores assigned by some subjects under time stress. Figs. 1 and 2 plot the mean scores against sugar concentration and against percent of higher-quality wine in the blend. For the sake of simplicity we have made the assumption that a linear relation exists between the objective and subjective scales. For the quantity evaluation this difference was con-

Table 1. Analysis of red sweet wines used in the quality experiment.

Category	Total acid (g H_2Ta/100 ml)	Volatile acid (g HOAc/100 ml)	pH	Extract (g/100 g)	Reducing sugar (g/100 ml)	Ethyl alcohol (% by volume)	Tannin (g/100 ml)	Color[a] Duboscq
Good	0.50	0.02	3.91	14.0	10.6	19.6	0.20	360
Poor	0.69	0.05	3.39	12.8	10.8	20.4	0.06	29

[a] Increasing values indicate increasing color (on a color density basis).

Table 2. Mean (ten replications) and grand mean scores by individual tasters for each sugar level with and without time stress for the quantity evaluation.

Tasters	Sugar level 1	2	3	4	5	6	7	8	9	10	Grand means
Normal time											
1	1.6	2.2	2.1	2.7	4.3	5.9	6.8	7.8	8.6	9.0	5.10
2	1.3	1.6	2.1	3.3	4.5	4.6	6.1	7.1	9.2	9.1	4.89
3	1.5	1.8	2.3	3.4	5.1	6.0	6.9	7.2	7.6	8.4	5.02
4	1.8	1.7	2.4	3.7	5.0	5.7	7.4	7.4	8.4	9.3	5.28
5	1.4	1.8	2.3	4.0	4.4	4.8	6.6	7.5	8.9	7.5	4.92
6	1.5	1.9	2.3	4.0	3.4	5.1	5.8	7.8	7.9	8.8	4.85
7	1.5	2.5	3.8	4.0	6.5	6.6	7.4	8.4	9.4	9.2	5.93
8	1.6	1.8	2.0	2.6	4.2	4.4	6.2	5.8	7.9	8.5	4.50
9	2.4	2.1	2.3	3.2	3.7	4.3	5.0	7.1	6.3	7.6	4.40
10	1.3	1.3	2.6	3.1	4.8	5.0	6.9	7.6	7.5	8.5	4.86
Grand means	1.59	1.87	2.42	3.40	4.59	5.24	6.51	7.37	8.17	8.59	4.98
Stressed time											
1	1.7	2.8	2.9	4.5	6.3	6.6	8.0	8.8	9.5	9.6	6.07
2	1.5	1.8	2.0	3.0	3.8	4.7	6.3	6.7	7.7	8.6	4.61
3	1.9	2.0	3.0	4.7	5.9	7.0	7.8	8.0	8.7	9.6	5.86
4	1.3	2.3	2.7	4.1	4.5	5.4	6.8	7.2	8.3	8.6	5.12
5	1.5	2.9	2.8	3.8	5.4	5.2	6.4	7.5	7.1	7.3	4.99
6	2.0	2.1	3.4	4.3	4.5	7.0	6.3	7.7	8.5	9.3	5.51
7	1.6	1.9	3.8	5.5	7.1	7.5	8.5	8.9	9.5	9.5	6.38
8	1.1	1.6	1.9	2.8	4.4	4.7	5.4	5.8	8.6	8.4	4.47
9	2.6	3.2	2.8	3.8	4.9	5.1	5.4	6.7	7.9	8.3	5.07
10	1.9	2.3	3.3	3.9	4.7	5.4	6.7	7.3	8.0	8.1	5.16
Grand means	1.71	2.29	2.86	4.04	5.15	5.86	6.76	7.46	8.38	8.73	5.32

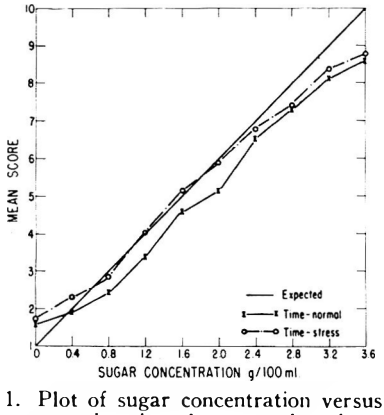

Fig. 1. Plot of sugar concentration versus mean total scores assigned under normal and stressed-time conditions.

sistent over the whole scale, whereas with the quality evaluation the differences were not seen at the high end of the scale. Individual plots of the subjects indicated clearly that some subjects consistently assigned higher scores under the time-stress conditions—in both cases—whereas others showed no tendency to do this. This is shown in Table 4, which compares the individual grand means for the two experiments and also shows the actual average time used by the various subjects in the *ad libitum* tasting. These measurements were made on the concluding tests of the quality study. Records

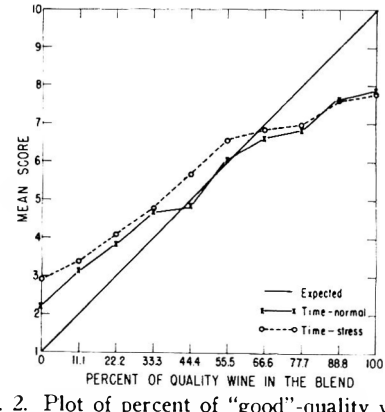

Fig. 2. Plot of percent of "good"-quality wine in the blend versus mean total scores assigned under normal and stressed-time conditions.

Table 3. Mean (ten replications) and grand mean scores by individual tasters for each blend with and without time-stress quality evaluation.

Tasters	Blends 1	2	3	4	5	6	7	8	9	10	Grand means
Normal time											
1	1.7	1.8	2.6	3.3	3.9	4.8	6.6	6.1	6.9	7.0	4.47
2	2.3	3.1	3.4	3.8	4.9	6.4	7.1	6.6	8.6	6.7	5.29
3	1.2	3.0	2.9	4.7	3.3	4.9	4.9	5.6	7.2	8.3	4.60
4	2.4	2.1	3.1	3.0	4.7	5.2	6.8	7.5	8.4	7.6	5.08
5	2.5	2.9	3.2	6.4	6.6	7.4	6.3	7.4	6.4	8.1	5.72
6	1.8	2.9	4.4	4.8	4.6	6.4	7.2	8.0	8.0	7.2	5.53
7	3.6	5.2	5.0	5.4	5.7	6.9	8.3	7.8	7.4	7.9	6.32
8	3.8	6.0	7.3	6.8	5.3	7.5	6.1	4.6	7.8	8.8	6.40
9	1.0	1.9	2.9	4.2	4.7	6.1	6.6	7.5	8.2	9.0	5.21
10	1.3	2.2	3.3	4.3	4.5	4.8	6.1	7.2	7.6	8.2	4.95
Grand means	2.16	3.11	3.81	4.67	4.82	6.04	6.60	6.83	7.65	7.88	5.35
Stressed time											
1	3.2	2.4	3.7	3.6	4.7	6.3	6.2	6.8	8.2	9.0	5.41
2	2.7	5.0	4.4	4.5	6.6	8.1	6.2	4.4	7.4	6.6	5.59
3	1.7	2.2	3.3	4.2	5.0	5.6	6.2	7.7	7.5	8.0	5.14
4	2.9	2.2	3.4	3.5	6.6	6.5	7.7	7.2	9.0	7.8	5.68
5	2.8	3.6	4.9	2.8	4.4	5.8	5.3	6.2	4.4	6.1	4.63
6	2.2	2.3	4.1	5.8	5.1	6.2	6.9	7.5	8.0	7.3	5.54
7	5.2	5.2	4.0	6.7	7.2	5.6	8.4	9.1	8.3	8.1	6.78
8	3.8	5.2	5.0	5.7	5.9	8.5	7.2	5.8	8.1	7.8	6.30
9	2.2	3.0	4.3	6.3	5.3	7.9	7.5	7.7	8.4	9.2	6.18
10	1.8	2.2	3.8	4.6	6.2	5.0	6.5	7.3	7.1	8.0	5.25
Grand means	2.85	3.33	4.09	4.77	5.70	6.55	6.81	6.97	7.64	7.79	5.65

were kept throughout the study on the times that a taster went over the allowable stress time. For the quantity study, taster 5 went over the time limit once and taster 7 four times. With the quality study, taster 4 went over once and taster 7 once. Taster 2 could not make the decisions in the required time for either of the studies. There appears to be little correlation between the time used and the grand mean times. The subjects who had the greatest difference in mean scores were not those who had the greatest difference between normal and stress times. Many of the subjects used less than the

Table 4. Effect of time stress on tasters' grand mean scores with quantity and quality studies.

Taster	Time (sec used, av.) Normal	Stress	Quantity Time (mean scores) Normal	Stress	Significance level (%)	Quality Time (mean scores) Normal	Stress	Significance level (%)
1	11.0	6.5	5.10	6.07	1.0	4.47	5.41	1.0
2	31.0	11.0	4.89	4.61	.....	5.29	5.59	.....
3	7.5	6.0	5.02	5.86	1.0	4.60	5.14	5.0
4	7.0	6.0	5.28	5.12	.....	5.08	5.68	.....
5	10.0	7.0	4.92	4.99	.....	5.72	4.63	.....
6	8.5	8.0	4.85	5.51	1.0	5.53	5.54	.....
7	18.5	8.0	5.93	6.38	.....	6.32	6.78	.....
8	7.5	7.5	4.50	4.47	.....	6.40	6.30	.....
9	10.5	6.5	4.40	5.07	1.0	5.21	6.18	1.0
10	8.0	6.5	4.86	5.16	5.0	4.95	5.25	.....
Total	11.93	6.75	4.975	5.324		5.357	5.650	

specified time under normal conditions, but said that under the stop watch they felt rushed.

There seems to be a definite tendency for some subjects to assign higher scores under the stress condition. This cannot be explained as a general central tendency reaction. The distribution of the scoring, or scale used, was investigated. Tables 5 and 6 show the data and roughly indicate the general agreement or disagreement with the expected distribution. A tendency for overall bimodal distribution was indicated. Fig. 3 shows a graph of total distribution under stressed time and normal conditions and verifies the bimodality. The bimodal situation is probably the result of the use of two reference samples and the subjects' tendency to not move too far from the reference sample that was closest in composition or quality.

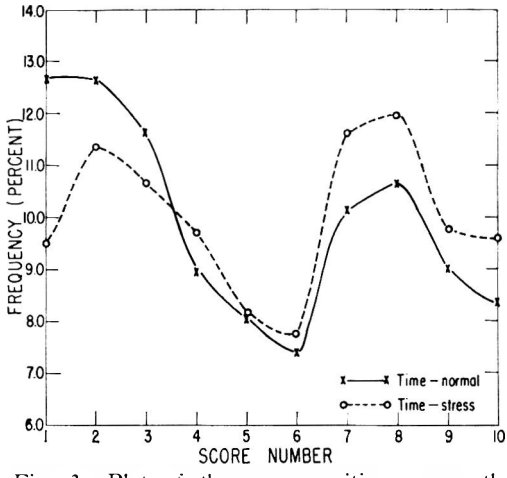

Fig. 3. Plot of the score position versus the frequency of expected score assignment for all normal and all stressed-time scores.

These data appear to verify Brunswick's theory in our interpretation—that under "perceptual" conditions (stressed time) the quantity scores were better distributed about the expected values, and the mean values were closer to the expected means. With the quality situation, the reverse appears to

Table 5. Distribution of quantity scores with respect to whole score range under normal and stressed-time conditions.

	Score position										Distribution[a] check
Taster	1	2	3	4	5	6	7	8	9	10	χ^2, 7 *df*
Normal time											
1	14	12	12	10	8	6	9	11	11	7	5.6
1	15	17	11	11	7	3	7	10	9	10	14.4*
3	11	15	12	8	11	7	10	12	10	4	8.4
4	12	15	13	7	5	5	11	10	13	9	18.8**
5	19	14	10	8	8	5	4	17	1	14	31.2***
6	11	19	13	8	8	6	9	12	10	4	15.6*
7	11	10	10	7	4	6	12	11	14	15	10.8
8	17	17	11	11	7	9	9	8	4	7	16.0*
9	9	18	17	14	10	7	12	8	2	3	26.0***
10	16	15	8	8	9	8	12	11	11	2	14.4*
Total	135	152	117	92	77	62	95	110	85	75	72.30***
Stressed time											
1	6	15	9	6	5	9	9	8	16	17	17.4*
2	14	15	17	10	11	4	6	7	11	5	17.8*
3	9	10	10	7	9	6	9	18	9	13	10.2
4	13	10	13	9	10	9	12	9	6	9	4.2
5	17	8	12	8	9	11	12	12	3	8	12.4
6	9	11	11	8	10	5	14	17	9	6	11.4
7	10	10	8	6	4	5	8	13	10	26	35.0***
8	24	11	10	10	8	9	9	4	8	7	25.2***
9	8	11	11	11	15	12	14	9	7	2	12.6
10	3	15	14	13	11	8	12	14	10	0	22.4***
Total	113	116	115	88	92	78	105	111	89	93	16.58*

[a] Using the expected value of 10 for each score position for each individual and 100 for total expected for each score position.

Table 6. Distribution of quality scores with respect to whole score range under normal and stressed-time conditions.

Taster	Score position 1	2	3	4	5	6	7	8	9	10	Distribution[a] check χ^2, 7*df*
Normal time											
1	14	12	17	11	12	12	7	6	5	4	16.4*
2	12	11	12	9	8	10	10	8	11	9	2.0
3	13	15	12	13	11	11	10	2	8	5	14.2*
4	16	16	13	3	11	5	5	5	11	15	23.2**
5	13	7	10	9	7	7	11	14	7	15	8.8
6	8	7	13	10	11	7	14	16	11	3	13.4
7	6	7	8	6	8	10	16	13	12	14	11.4
8	10	5	6	6	9	8	9	16	16	15	16.0*
9	16	10	9	9	8	7	13	12	7	9	7.4
10	10	11	16	11	9	9	13	11	7	3	10.8
Total	118	101	116	87	94	86	108	103	95	92	11.44
Stressed time											
1	10	8	10	16	10	7	11	9	10	9	5.2
2	6	10	11	10	12	12	8	15	8	8	6.2
3	10	15	11	11	9	8	7	14	7	8	7.0
4	12	19	7	7	3	2	8	10	14	18	30.0***
5	15	8	14	13	13	11	11	8	4	3	15.4*
6	5	11	15	10	7	8	14	13	13	4	13.4
7	2	9	9	8	4	6	10	15	17	20	29.6***
8	5	7	5	11	12	6	14	14	14	12	13.2
9	5	10	6	12	9	5	11	17	11	14	14.5*
10	7	14	10	8	12	12	14	12	8	3	11.0
Total	77	111	98	106	91	77	108	127	106	99	21.30**

[a] Using the expected value of 10 for each score position for each individual and 100 for total expected for each score position.

be true—that is, the score distribution under normal or "intellectual" conditions is superior and the scoring follows expected scores more closely. In this latter case the differences are small but definite, as shown in Fig. 2.

It was of interest to investigate the replicates and determine the total variances for the subjects and for the levels. Tables 7 and 8 show these data and the sums of squares of deviations from each individual replicate mean. The variances among the individual judges differ quite noticeably. Taster 5 in particular had a large variance in the quantity study. Tasters in general had consistent variances within a study. Variances were larger for the quality study because of the increased complexity of the task. Checking the rank correlation of the total replicate variance of the individuals between the quality and quantity study gave a correlation of +0.75. This was significant at the 2% level, indicating that the subjects' abilities to replicate scores were very similar between the two studies. Only three tasters (4, 5, and 9) changed more than two ranks; the biggest change was with taster 9. This taster was ranked 6 in the size of his variance in the quantity study, and was ranked 2 in the quality study.

The sugar level and blend quality variances increased toward the middle of the scale and were lower at the ends. This was owing to two factors: 1) the further the sample is from a reference the greater will be the variability of the score; and 2) in any closed scoring system, as the ends of the score range are approached the less scoring leeway is available by the nature of the system. These factors are also demonstrated in the work of Hanson *et al.* (1951). The results are in Table 9 for the individual analyses of variance for each subject for each study. Replicates were significantly different in several instances, indicating, most probably, changes in subjects' scoring standards

Table 7. Sums of squares of deviations from the

					Sugar
Tasters	1	2	3	4	5
Normal time					
1	6.40	7.60	8.90	18.10	12.10
2	2.10	8.40	6.90	14.10	28.50
3	2.50	5.60	8.10	10.40	26.90
4	15.60	4.10	8.40	26.10	52.00
5	4.40	5.60	16.10	34.00	64.40
6	4.50	4.90	8.10	40.00	18.40
7	4.50	14.50	59.60	36.00	48.50
8	8.40	9.60	12.00	10.40	45.60
9	14.40	4.90	8.10	25.60	12.10
10	4.10	2.10	6.40	28.90	29.60
Variance	0.743	0.747	1.584	2.706	3.756
df	90	90	90	90	90
Stressed time					
1	2.10	15.60	18.90	36.50	34.10
2	4.50	7.60	6.00	12.00	11.60
3	4.90	10.00	8.00	36.10	22.90
4	2.10	8.10	34.10	8.90	18.50
5	8.50	30.90	13.60	63.60	48.40
6	14.00	14.90	22.40	22.10	24.50
7	4.40	8.90	39.60	28.50	50.90
8	0.90	10.40	8.90	27.60	24.40
9	14.40	19.60	19.60	23.60	32.90
10	2.90	6.10	4.10	10.90	16.10
Variance	0.652	1.467	1.946	2.997	3.158
df	90	90	90	90	90

replicate means and associated variances for quantity study.

evels						
6	7	8	9	10	Variance	*df*
10.90	37.60	21.60	8.40	6.00	1.528	90
46.40	44.90	22.90	9.60	8.90	2.141	90
32.00	20.90	25.60	38.40	10.40	2.008	90
38.10	42.40	22.40	26.40	6.10	2.684	90
79.60	64.40	40.50	32.90	54.50	4.404	90
18.90	25.60	23.60	28.90	3.60	1.961	90
30.40	34.40	24.40	6.40	5.60	2.936	90
18.40	33.60	29.60	42.90	22.50	2.588	90
38.10	34.00	32.90	28.10	16.40	2.384	90
12.00	22.90	26.40	8.50	14.50	1.726	90
3.608	4.007	2.998	2.561	1.650	2.436	
90	90	90	90	90		900
26.40	22.00	13.60	4.50	2.40	1.956	90
22.10	38.10	40.10	26.10	42.40	2.339	90
38.00	19.60	6.00	10.10	4.40	1.777	90
36.40	49.60	30.60	16.10	18.40	2.475	90
49.60	50.40	22.50	68.90	48.10	4.494	90
18.00	26.10	6.10	4.50	6.10	1.763	90
54.50	24.50	12.90	4.50	8.50	2.635	90
38.10	42.40	49.60	14.40	30.40	2.745	90
34.90	28.40	16.10	16.90	10.10	2.405	90
20.40	20.10	12.10	14.00	6.90	1.262	90
3.760	3.568	2.328	2.000	1.974	2.385	
90	90	90	90	90		900

Table 8. Sums of squares of deviations from the replicate means and associated variances for the quality studies.

	Blends											
Tasters	1	2	3	4	5	6	7	8	9	10	Variance	*df*
Variance												
1	6.10	11.60	10.40	30.10	16.90	23.60	28.40	32.90	34.90	66.00	2.898	90
2	14.10	52.90	40.40	43.60	76.90	44.40	60.90	66.40	26.40	48.10	5.267	90
3	1.60	42.00	18.90	34.10	18.10	50.90	50.90	44.40	31.60	14.10	3.406	90
4	14.40	8.90	46.90	38.00	104.10	89.60	69.60	86.50	48.40	62.40	6.320	90
5	16.50	56.90	33.60	98.40	50.40	78.40	28.10	58.40	82.40	48.90	6.133	90
6	11.60	16.90	42.40	33.60	18.40	24.40	43.60	16.00	18.00	37.60	2.916	90
7	48.40	87.60	44.00	52.40	64.10	58.90	34.10	47.60	48.40	52.90	5.982	90
8	99.60	94.00	76.10	61.60	46.10	18.50	100.90	72.40	43.60	31.60	7.160	90
9	0.00	8.90	14.90	35.60	32.10	20.90	76.40	10.50	11.60	14.00	2.498	90
10	4.10	5.60	8.10	34.10	18.50	19.60	48.90	29.60	8.40	21.60	2.205	90
Variance	2.404	4.281	3.729	5.127	4.951	4.768	6.020	5.163	3.930	4.413	4.479	
df	90	90	90	90	90	90	90	90	90	90		900
Stressed time												
1	41.60	28.40	24.10	24.40	34.10	24.10	59.60	61.60	31.60	12.00	3.794	90
2	32.10	68.00	74.40	36.50	34.40	18.90	63.60	22.40	48.40	66.40	5.167	90
3	6.10	13.60	24.10	27.60	54.00	70.40	49.60	26.10	54.50	32.00	3.977	90
4	60.90	33.60	84.40	42.50	65.80	104.50	96.50	65.60	14.00	55.60	6.926	90
5	23.60	40.40	106.90	37.60	50.40	51.60	24.10	55.60	36.40	52.90	5.327	90
6	5.60	12.10	33.00	31.60	40.90	55.60	28.90	26.50	30.00	54.10	3.536	90
7	73.60	61.60	48.00	84.10	67.60	78.40	30.40	6.90	36.10	42.90	5.884	90
8	57.60	73.60	62.00	44.10	42.90	10.50	51.60	63.60	28.90	71.60	5.626	90
9	13.60	18.00	54.10	50.10	44.10	18.90	18.50	28.10	14.40	21.60	3.126	90
10	5.60	19.60	19.60	52.40	47.60	18.00	16.50	8.10	38.90	18.00	2.714	90
Variance	3.558	4.098	5.895	4.787	5.352	5.010	4.881	4.049	3.702	4.745	4.607	
df	90	90	90	90	90	90	90	90	90	90		900

Table 9. Comparison of mean square values calculated for each taster under each test condition.

	Quantity						Quality					
	Normal			Stress			Normal			Stress		
Tasters	Sugar levels	Replicates	S×R[a]	Sugar levels	Replicates	S×R	Blend levels	Replicates	B×R[b]	Blend levels	Replicates	B×R
1	82.60	2.66	1.41	86.71	1.87	1.97	44.00	3.53	2.83	49.63	4.68	3.70
2	85.45	4.79	1.85	67.03	6.28	1.90	43.17	1.79	5.65	27.45	4.52	5.24
3	66.79	4.40	1.74	79.78	3.69	1.59	44.82	3.93	3.35	51.56	5.34	3.83
4	78.73	3.88	2.55	65.86	2.20	2.52	56.28	8.20	6.11	59.13	2.24	7.38
5	69.00	7.93	4.01	45.60	8.39	4.06	42.24	7.40	5.99	15.98	4.25	5.57
6	70.92	4.29	1.70	68.70	3.17	1.61	46.71	5.25	2.66	43.63	4.29	3.43
7	79.36	4.22	2.79	91.60	0.91	2.83	24.15	3.88	6.22	29.28	8.20	5.63
8	64.22	2.78	2.57	71.31	5.27	2.47	23.73	3.51	7.57	23.40	5.07	5.69
9	41.27	2.71	2.35	41.78	2.11	2.44	73.97	3.34	2.41	57.04	3.97	3.03
10	72.74	2.83	1.60	53.09	2.98	1.07	53.58	4.14	1.99	46.05	3.54	2.62
df	9	9	81	9	9	81	9	9	81	9	9	81

[a] Sugar levels × replicate interactions.
[b] Blend levels × replicate interactions.

with time or possibly order effects. More replicate effects were noted in the quantity study, which was conducted first. Since the same order was used with both studies, this indicates that the effect was due primarily to subject adaption to the experiment. The sugar level × replicate interaction for the tasters under normal and stress conditions and the sugar level F values were correlated for the quantity study. The r value for the first was 0.95 and for the second 0.83. A similar correlation for the quality study gave r values of 0.90 and 0.94 for the tasters with blend levels × replicate interaction and for the F value when correlated between normal and stress conditions. Correlation of tasters F values between quantity and quality studies under normal and under stress conditions yield low r values, respectively 0.31 and 0.43. The low values are due primarily to tasters 2 and 9.

There is some question as to use of the analysis of variance with these data. One of the criteria for use is that the variance about the mean values should be homogeneous. Using Barlett's test (as described by Alder and Roessler, 1961) it was found that the variances for the replicates of the tested levels for both time conditions were homogeneous for the quality test but that the similar variances for the quantity test were significantly different at the 0.1% level. As stated by Amerine *et al.* (1962), the analysis of variance can be of value in indicating the nature of the data even though not all the criteria for exact interpretation of the results are met.

The use of analysis of variance in these cases would give only approximate results as far as evaluating true differences between levels. However, the interaction error term was used to compute significant differences. Since comparison of the method was all that was sought, not an exact measure of panel sensitivity, this use was thought valid. Table 10 compares the number of independent groupings that could be made by each subject under both conditions with both quantity and quality studies. The method for determining the significant differences was that of Duncan (1955). Some criterion must be formulated on which to base the

Table 10. The number of independent groupings[a] into which subjects could differentiate the wines.

	Quantity		Quality	
Taster	Normal	Stress	Normal	Stress
1	5	5	3	3
2	5	4	2	2
3	4	5	3	3
4	4	4	3	2
5	3	2	2	2
6	3	5	3	3
7	4	5	2	2
8	4	4	2	2
9	3	4	5	3
10	5	5	5	3

[a] S×R and B×R values of Table 9 used to calculate $S_{\bar{x}}$ term.

decision of which condition, normal or stress, gave the better results. It was decided that the number of independent groupings that could be made would be that criterion. The more independent groups, the better the discrimination, and hence the more desirable. The total number of groups represents the number of groups that can be determined. The number of independent groups represents the number of groups that the wines can be divided into and at least one wine of each group be significantly different from the other groups. Arbitrarily, the mean scores were lined up in order of expected mean score. In cases where reversals in mean scores arose, the out-of-place mean score was included in its bracketing group although it may have been significantly different. This was considered justified in this instance because the question and condition of the test instructed the subjects in what direction to score the wines. On an individual basis, with the quantity study, better grouping was achieved under the time-stress conditions by four of the ten subjects. Two subjects grouped better under normal conditions. The rest of the subjects responded equally to the two time conditions. With the quality study three subjects grouped the wine more successfully under the normal condition and the rest of the subjects grouped them equally well under both conditions.

To determine the over-all panel response to the two conditions the panel mean scores were tested in a similar manner. The combined replicate variances of the deviations from the individual means were used to calculate significant differences. Again, because of use of significant differences as only a method of comparison and of the general similarity between replicate variances of the deviations from the individual means and the calculated error terms from the analysis of variance, this approximation was made. This error term would not include taster interactions. Table 11 gives the mean scores and shows that, for the quantity study, the panel could group the ten wines into eight independent groups under normal conditions, and into nine under stress conditions. Conversely with the quality study, the wines could be grouped into seven groups under normal conditions, and into only six under stress conditions.

Table 11. Significant differences in mean scores for normal and stressed time conditions and the combined mean scores of the ten tasters.

Time	$S_{\bar{x}}$[a]										
Quantity study											
Normal	0.156	1.59	1.87[b]	2.42	3.40	4.59	5.15	6.51	7.37	8.17	8.59
Stress	0.154	1.71	2.29	2.86	4.04	5.15	5.86	6.76	7.46	8.38	8.73
Combined	0.110	1.65	2.08	2.64	3.72	4.87	5.50	6.64	7.42	8.27	8.66
Quality study											
Normal	0.212	2.16	3.11	3.81	4.67	4.82	6.02	6.60	6.88	7.65	7.88
Stress	0.215	2.85	3.33	4.09	4.77	5.70	6.55	6.81	6.97	7.64	7.79
Combined	0.151	2.50	3.22	3.95	4.72	5.26	6.28	6.71	6.90	7.64	7.84

[a] Standard error using the replicate variances calculated about the individual means (see Tables 7 and 8).

[b] Values underlined by same line are not significantly different at the 5% level.

Ough and Baker (1964) showed that subjects using intensity scales and paired-comparison techniques are essentially the same in their responses to within a linear transformation. The technique used to make this matter clear was correlation matrices, and the extraction of a single factor corresponding to the built-in structure of the experiment; see Cattell (1952). The levels were somewhat individual for some values.

These analyses of the data in this report are presented in Tables 12–19, which give

Table 12. Quantity study—normal time; correlation matrix for sugar levels.

Sugar levels	1	2	3	4	5	6	7	8	9	10
1	*(.70)*	.37	−.10	−.15	−.30	−.45	−.51	−.18	−.66	−.34
2	.37	(.60)	.50	.18	.28	.49	.01	.39	.20	.10
3	−.10	.50	(.80)	.52	.80	.64	.48	.66	.35	.28
4	−.15	.18	.52	(.70)	.27	.32	.20	.61	.35	.00
5	−.30	.28	.80	.27	*(1.00)*	.74	.97	.41	.53	.42
6	−.45	.49	.64	.32	.74	(.80)	.78	.67	.44	.56
7	−.51	.01	.48	.20	.97	.78	(1.00)	.40	.60	.53
8	−.18	.39	.66	.61	.41	.67	.40	(.70)	.36	.28
9	−.66	.20	.35	.35	.53	.44	.60	.36	(.70)	.54
10	−.34	.10	.28	.00	.42	.56	.53	.28	.54	*(.60)*
Total	3.02	2.38	5.13	3.30	5.72	5.89	5.48	4.66	4.73	3.65
F_1	−.46	.36	.77	.50	.86	.89	.83	.70	.71	.55

Correlation coefficients calculated from data in Table 2 (normal time).

Table 13. Quantity study—stressed time; correlation matrix for sugar level.

Sugar levels	1	2	3	4	5	6	7	8	9	10
1	*(.60)*	.56	.42	.36	.12	.22	−.09	.15	−.08	.11
2	.56	(.80)	.18	.11	.17	.75	−.11	.16	−.28	−.33
3	.42	.18	(.90)	.88	.64	.79	.57	.76	.38	.36
4	.36	.11	.88	(.90)	.83	.90	.83	.89	.61	.61
5	.12	.17	.64	.83	*(.90)*	.72	.79	.80	.62	.46
6	.22	.75	.79	.90	.72	(.90)	.76	.83	.69	.77
7	−.09	−.11	.57	.83	.79	.76	(.90)	.88	.65	.63
8	.15	.16	.76	.89	.80	.83	.88	*(.90)*	.57	.58
9	−.08	−.28	.38	.61	.62	.69	.65	.57	(.90)	.86
10	.11	−.33	.36	.61	.46	.77	.63	.58	.86	(.90)
Total	2.37	2.01	5.58	6.92	6.05	7.33	5.81	6.52	4.92	4.95
F_1	.33	.28	.81	.95	.83	1.01	.80	.90	.68	.68

Correlation coefficients calculated from data in Table 2 (stressed time).

Table 14. Quality study—normal time; correlation matrix for quality blend levels.

Blends	1	2	3	4	5	6	7	8	9	10
1	(.90)	.85	.79	.56	.64	.71	.46	−.21	−.03	.01
2	.85	*(.95)*	.91	.74	.42	.69	.30	−.35	−.03	.27
3	.79	.91	(.95)	.70	.37	.67	.20	−.39	.13	.30
4	.56	.74	.70	(.85)	.61	.79	−.09	−.27	−.43	.51
5	.64	.42	.37	.61	*(.85)*	.83	.46	.31	−.20	.14
6	.71	.69	.67	.79	.83	(.85)	.37	.00	−.05	.21
7	.46	.30	.20	−.09	.46	.37	(.65)	.60	.27	−.40
8	−.21	−.35	−.39	−.27	.31	.00	.60	*(.65)*	−.12	−.22
9	−.03	−.03	.13	−.43	−.20	−.05	.27	−.12	(.50)	−.14
10	.01	.27	.30	.51	.14	.21	−.40	−.22	−.14	(.55)
Total	5.16	5.51	5.15	5.37	4.21	5.17	1.08	1.30	1.10	1.95
F_1	.86	.92	.86	.90	.70	.86	.18	−.22	−.18	.33

Correlation coefficients calculated from data in Table 3 (normal time).

Table 15. Quality study—stressed time; correlation matrix for quality blend levels.

Blends	1	2	3	4	5	6	7	8	9	10
1	(.80)	.71	.27	.34	.48	.12	.54	.18	.21	.04
2	.71	(.80)	.66	.39	.47	.50	.23	−.31	−.07	−.30
3	.27	.66	(.70)	.17	−.13	.53	−.18	−.50	−.59	−.44
4	.34	.39	.17	(.80)	.41	.27	.70	.42	.50	.38
5	.48	.47	−.13	.41	(.70)	.10	.68	.09	.47	.01
6	.12	.50	.53	.27	.10	(.60)	.15	−.58	.29	.03
7	.54	.23	−.18	.70	.68	.15	(.80)	.57	.76	.48
8	.18	−.31	−.50	.42	.09	−.58	.57	(.60)	.32	.53
9	.21	−.07	−.59	.50	.47	.29	.76	.32	(.70)	.68
10	.04	−.30	−.44	.38	.01	.03	.48	.53	.68	(.70)
Total	3.15	1.76	.91	4.04	3.54	.95	5.09	2.32	4.45	2.99
F_1	.58	.33	−.17	.75	.66	.18	.94	.43	.82	.55

Correlations coefficients calculated from data in Table 3 (stressed time).

Table 16. Quantity study—normal time; correlation matrix for tasters.

Tasters	1	2	3	4	5	6	7	8	9	10
1	(1.00)	.98	.98	.98	.97	.97	.96	.98	.97	.98
2	.98	(1.00)	.96	.83	.89	.88	.86	.90	.85	.88
3	.98	.96	(1.00)	.99	.96	.96	.98	.97	.95	.99
4	.98	.83	.99	(1.00)	.97	.97	.97	.99	.96	.99
5	.97	.89	.96	.97	(1.00)	.96	.96	.95	.94	.97
6	.97	.88	.96	.97	.96	(1.00)	.94	.95	.98	.96
7	.96	.86	.98	.97	.96	.94	(1.00)	.95	.92	.98
8	.98	.90	.97	.99	.95	.95	.95	(1.00)	.94	.98
9	.97	.85	.95	.96	.94	.98	.92	.94	(1.00)	.96
10	.98	.88	.99	.99	.97	.96	.98	.98	.96	(1.00)
Total	9.77	9.03	9.74	9.61	9.57	9.57	9.52	9.61	9.47	9.68
F_1	1.00	.92	.99	.98	.98	.98	.97	.98	.97	.99

Correlation coefficients calculated from data in Table 2 (normal time).

Table 17. Quantity study—stressed time; correlation matrix for tasters.

Tasters	1	2	3	4	5	6	7	8	9	10
1	(1.00)	.98	.99	.99	.99	.96	.98	.97	.96	.99
2	.98	(1.00)	.97	.98	.95	.96	.93	.97	.97	.98
3	.99	.97	(1.00)	.98	.97	.98	.99	.96	.95	.98
4	.99	.98	.98	(1.00)	.96	.97	.96	.97	.96	.99
5	.99	.95	.97	.96	(1.00)	.93	.97	.93	.94	.97
6	.96	.96	.98	.97	.93	(1.00)	.95	.96	.96	.97
7	.98	.93	.99	.96	.97	.95	(1.00)	.93	.91	.97
8	.97	.97	.96	.97	.93	.96	.93	(1.00)	.99	.97
9	.96	.97	.95	.96	.94	.96	.91	.99	(1.00)	.96
10	.99	.98	.98	.99	.97	.97	.97	.97	.96	(1.00)
Total	9.81	9.69	9.77	9.76	9.71	9.64	9.59	9.65	9.60	9.78
F_1	1.00	.98	.99	.99	.99	.98	.97	.98	.97	.99

Correlation coefficients calculated from data in Table 2 (stressed time).

the first factor weights (F_1 values) calculated from the correlation matrices for Tables 2 and 3 going both ways in each table subtable. The size of F_1 for a particular test position or for a particular taster indicates the over-all connection of a test position or taster with all the other test positions or tasters, respectively. The determinability of the test position or taster is measured by the square of F_1, and the uniqueness or independence of the other related variables is measured by 1 − (square of F_1) (see Baker and Baker, 1956). The diagonal terms in the correlation matrices in

Table 18. Quality study—normal time; correlation matrix for tasters.

Tasters	1	2	3	4	5	6	7	8	9	10
1	(1.00)	.96	.89	.98	.83	.96	.95	.45	.98	.97
2	.96	(1.00)	.84	.96	.78	.95	.92	.46	.94	.91
3	.89	.84	(.95)	.85	.81	.87	.84	.69	.94	.94
4	.98	.96	.85	(1.00)	.77	.94	.90	.36	.96	.95
5	.83	.78	.81	.77	(.95)	.83	.81	.42	.89	.85
6	.96	.95	.87	.94	.83	(1.00)	.95	.47	.96	.95
7	.95	.92	.84	.90	.81	.95	(1.00)	.43	.93	.91
8	.45	.46	.69	.36	.42	.47	.43	(.60)	.53	.51
9	.98	.94	.94	.96	.89	.96	.93	.53	(1.00)	.99
10	.97	.91	.94	.95	.85	.95	.91	.51	.99	(1.00)
Total	8.97	8.72	8.62	8.67	7.94	8.88	8.64	4.92	9.12	8.98
F_1	.98	.95	.94	.95	.87	.97	.95	.54	1.00	.98

Correlation coefficients calculated from data in Table 3 (normal time).

Table 19. Quality study—stressed time; correlation matrix for tasters.

Tasters	1	2	3	4	5	6	7	8	9	10
1	(1.00)	.63	.94	.92	.73	.88	.73	.80	.91	.89
2	.63	(1.00)	.61	.71	.52	.60	.34	.92	.70	.59
3	.94	.61	(1.00)	.92	.75	.96	.85	.76	.95	.97
4	.92	.71	.92	(1.00)	.68	.89	.81	.79	.87	.91
5	.73	.52	.75	.68	(.80)	.65	.44	.61	.70	.71
6	.88	.60	.96	.89	.65	(1.00)	.81	.78	.96	.93
7	.73	.34	.85	.81	.44	.81	(.90)	.49	.74	.85
8	.80	.92	.76	.79	.61	.78	.49	(1.00)	.87	.70
9	.91	.70	.95	.87	.70	.96	.74	.87	(1.00)	.91
10	.89	.59	.97	.91	.71	.93	.85	.70	.91	(1.00)
Total	8.43	6.62	8.71	8.50	6.59	8.46	6.96	7.72	8.61	8.46
F_1	.95	.74	.98	.96	.74	.95	.78	.87	.97	.95

Correlation coefficients calculated from data in Table 3 (stressed time).

Tables 12–19 are assigned arbitrarily as being somewhat larger than any correlation coefficient in corresponding column, as suggested by Cattell (1952). No detailed discussion is given of the statistical significance of the elements of structure revealed by Tables 12–19, since some of the points made are already supported by the other analyses cited and the other findings are so striking that no tedious listing of the results of statistical tests is necessary.

The main points indicated in Table 12 for the quantity normal-time study concerning individual sugar levels were that the judges responded quite differently to the lowest sugar level than to the others, as was evidenced by the negative correlations with the other sugar levels for the 10 tasters. Levels 5, 6, and 7 were determined to about the same extent for all tasters, but the other positions were more unique. The sugar levels were determined more uniformly under the stressed-time condition than in the non-stressed condition, as is shown by the F_1 values. With the quality study under normal time conditions, blends 1–6 were determined uniformly by tasters but that 7–10 were quite unique. Under stress conditions, however, the quality judgments of the ten tasters were greatly disturbed (Table 15). The correlation coefficients within the matrix were low, and many of them negative. The F_1 values show quality blend level 7 to be determined to some extent, but blend levels 4 and 9 are determined to a lesser degree. It is indicated by the F_1 values in these four tables that the tasters responded much more uniformly to the quantity decisions under stress conditions and for the quality decisions more consistent values were assigned under normal test conditions.

Tables 16 and 17 give the data for the quantity study for normal and stressed-time conditions for the taster correlation matrices.

In both cases the coefficients of correlation are uniformly very high. Thus, stressing does not change the way the scales are used by the individual tasters except for a linear transformation, as pointed out previously. Also, as found by Ough and Baker, the tasters all used the scales almost identically except for change of origin and scale for quantity evaluation.

Table 18 refers to the normal-time quality-study data for the tasters and shows that in tasting for quality under unstressed conditions, taster 8 behaved in an entirely different manner from any of the other tasters, who all behaved very much alike.

Table 19 gives the correlation matrix for the tasters for the stressed-time quality test, and showed that quality responses are somewhat erratic and the scale use not as closely similar among tasters under stress as under normal conditions. The correlation coefficients now range from .34 to .97 instead of nearly all of them being above .9, as in Tables 16 and 17.

One further comparison is of interest. The correlations between tasters for unstressed and stressed conditions for quality and quantity responses were as follows:

Taster	Quantity	Quality
1	0.98	0.94
2	0.99	0.74
3	1.00	0.90
4	0.99	0.96
5	0.96	0.64
6	0.98	0.97
7	0.98	0.80
8	0.99	0.74
9	0.96	0.97
10	0.99	0.96

The correlations are much more consistent and higher for quantity ratings than for quality responses, indicating that the stress condition was much more upsetting to certain tasters for the quality study.

Also, results in Tables 10, 11, and 12–18 further justify the conclusions that, for quantity evaluation, time stress is advantageous, and for quality studies normal situations with no time stress are superior. We conclude that the interpretation of the Brunswick probabilistic theory as made in this report is generally verified to the extent of these data. Also, we conclude that tasters are the same except for a change of origin and scale unit on quantity ratings, but this is not necessarily so for quality evaluations. The determining blends vary greatly from situation to situation.

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Effects of Sample Sequence on Food Preferences[a,b]

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(Manuscript received August 26, 1963)

SUMMARY

Hedonic-scale preference tests were run with four types of meat. Each one compared two nonirradiated ("good") samples with two irradiated ("poor") samples. Three effects were demonstrated: a) position effect (the later samples in the series were rated lower); b) contrast effect (serving "good" samples first lowered the ratings for "poor" samples); and c) convergence effect (serving "poor" samples first lowered the ratings for "good" samples). Contrast and convergence effects were shown to be independent of position effect.

When several food samples are presented successively to be rated for preference, the first ones served tend to be liked better than the later ones. This phenomenon has often been observed in single-stimulus testing. Various explanations have been offered, of which the following appear to be most relevant:

1) *Position effect,* which is no more than reification of the observation. It is alleged that simply the fact that a food sample is served later in a series accounts for the lower preference. This may be due to fatigue, boredom, or the development of more critical attitudes based on greater experience with the samples.

2) *Contrast effect,* which is probably the best known of the three. When a better sample is served, followed by a poorer one, ratings of the poorer sample are further depressed, and differences between samples are increased. Contrast has been explained in terms of the general relativity of things; the good qualities of the better sample accentuate the shortcomings of the other. Contrast has been investigated and substantiated in different sensory modalities such as intensity and preference (Hanson *et al.,* 1956). Its existence and the ever-present possibility of its influencing test results are a sort of "common knowledge" to most people experienced in sensory testing.

3) *Convergence effect,* which is alleged to occur when a poor sample precedes and causes a good sample to be rated lower than it would have been otherwise. Kamenetzky (1959) first stated and explained the convergence hypothesis. This hypothesis states that, for many foods, all samples have common desirable and undesirable qualities, and detection of readily apparent poor qualities in the first (poor) sample will call special attention to the presence of small amounts of these same undesirable qualities in the better sample. Another explanation may be that the poor sample causes a generally negative attitude toward the food that persists during later presentations. Convergence effects were not found in the single case where they were systematically investigated (Kamenetzky, 1959).

Position effect *per se* is independent of food quality, in the sense that it can be

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[a] Presented at the 22nd Annual Meeting of the Institute of Food Technologists, Miami Beach, May, 1962.

[b] This paper reports research undertaken at the Armed Forces Food & Container Institute and has been assigned number 2283 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

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demonstrated when identical samples are served successively. Operationally, contrast and convergence require that the samples differ in quality. They also require prescribed serving orders—for contrast, the good-quality sample first; and for convergence, the opposite. With both, the lowering of preference is induced by comparison between samples.

The present study was made to investigate these phenomena systematically.

METHOD

All the tests were conducted under controlled conditions in the Sensory Evaluation Laboratory at the Armed Forces Food & Container Institute. The subjects were drawn at random from a pool of about 450 employees who had volunteered for the regular taste-test program and who also had expressed a willingness to test irradiated foods. The single-stimulus method was used, each subject receiving four samples at each session. Samples were rated on the 9-point hedonic scale (Peryam and Pilgrim, 1957).

Meats were selected as the material for investigation, with irradiation as the primary variable. One half of each lot of meat was preserved by freezing and the other half was subjected to 4.5-megarad irradiation. A second 2-category variable was included in each experiment so that there would be four samples for evaluation. Ham was obtained from two different processors; age (fryer vs. fowl) was the secondary variable for chicken; and the method of enzyme inactivation (steam heat vs. dry heat) varied for pork roast. None of these variables had any effect on preference ratings. Four experiments were run, all with the same design, as follows: 1) ham served at room temperature; 2) pork roast served at room temperature; 3) fried chicken, white meat, served hot; and 4) fried chicken, dark meat, served hot.

Five different serving-order plans were used with each type of meat. Each design required 24 subjects to rate four samples. In the following description of the designs, the subscripts refer to the two categories of the second variable. This variable served a purpose in establishing the designs, although in the analysis presented here the two samples were treated as replicates.

1) Conventional. Each of the 24 possible permutations of the 4 samples, 2 control (C_1, C_2) and 2 irradiated (I_1, I_2), was served once.

2) All control. Each of the 4 possible combinations of pairs of the two control samples was served six times: C_1-C_2-C_1-C_2, C_1-C_2-C_2-C_1, C_2-C_1-C_1-C_2, and C_2-C_1-C_2-C_1.

3) All irradiated. The same as the *all control*, but using the two irradiated samples.

4) Contrast. Each of the 4 possible combinations of pairs in which the two control samples precede the two irradiated samples was served six times: C_1-C_2-I_1-I_2, C_1-C_2-I_2-I_1, C_2-C_1-I_1-I_2, and C_2-C_1-I_2-I_1.

5) Convergence. The same as the contrast design, but with the irradiated samples preceding the controls.

For purposes of analysis, the usual practice was followed of assigning the integral values 1–9 to the categories of the hedonic scale, beginning with "dislike extremely," and average ratings were obtained. An analysis of variance was performed separately for each design and each type of meat. Where not otherwise stated, the significance levels of the differences between treatments were obtained from these analyses.

RESULTS

Tables 1–5 show the average rating of "treatments" obtained using the five different designs. Each mean is based on the data for two samples, either $C_1 + C_2$ or $I_1 + I_2$, a total of 48 ratings. This is true whether the variable is "control vs. irradiated" or "positions 1 & 2 vs. positions 3 & 4." Since the extraneous second variable, represented by the subscripts, had no detectable effect on preference, it could thus be treated as a pseudo-variable.

Table 1 presents the results for the *conventional* design. This is the design that would normally be used in such an experiment since it balances the four possible serving orders. These data confirm the expected effect of 4.5-megarad irradiation. For all four meat types the controls rated higher, with the significance level of the effects ranging from .01 to .06. Comparison of the results for the *all-control* design (Table 2) and the *all-irradiated* design (Table 3) gives further confirmation. The averages for the controls (Table 2) are higher than the comparable averages for the irradiated samples (Table 3) with one minor exception, that for *chicken, dark,* in positions 3 and 4. An analysis of variance on the combined data showed that the average difference between control and irradiated samples was significant at the .05 level.

Table 1. *Conventional* design. Mean preference for control and irradiated treatments.

Meat type	Control	Irradiated	Signif. of differences
Ham	7.17	6.46	.01
Pork	6.56	5.77	.01
Chicken, white	7.33	6.77	.02
Chicken, dark	6.75	6.06	.06
Av.	6.95	6.26	.001[a]

[a] Combined probability (Jones and Fiske, 1953).

Table 2. *All-control* design. Mean preference for samples according to order of serving.

Meat type	Positions 1 & 2	Positions 3 & 4	Signif. of differences
Ham	7.44	7.29	n.s.
Pork	6.58	6.83	n.s.
Chicken, white	7.52	7.10	.01
Chicken, dark	6.67	6.52	n.s.
Av.	7.05	6.93	n.s.

Thus, the general superiority of the control foods is demonstrated, even with the less efficient designs where the control and irradiated samples were not evaluated by the same individuals.

The primary reason for including *all-control* and *all-irradiated* designs was to test position effect uncontaminated with product differences. Results are presented in Tables 2 and 3. Comparison of the averages for the 1st and 2nd positions

Table 3. *All-irradiated design.* Mean preference for samples according to position of serving.

Meat type	Positions 1 & 2	Positions 3 & 4	Signif. of differences
Ham	7.17	6.90	n.s.
Pork	6.38	6.35	n.s.
Chicken, white	6.96	6.96	n.s.
Chicken, dark	6.48	6.58	n.s.
Av.	6.75	6.70	n.s.

combined, with those for the 3rd and 4th positions combined, failed to show the expected lower ratings for the later samples. There was only one significant trend toward higher preference for the earlier samples (*chicken, white,* in the *all-control* design), and in three of the eight cases the averages for the later samples were actually higher.

These data, however, are not conclusive evidence of absence of the position effect. These designs may have failed to demonstrate it because of the small number (24) of subjects. Therefore, position effect was further examined by combining all data from the *conventional, all-control,* and *all-irradiated* designs. This combining was such that each of the four samples appeared equally often in each position. The results (Table 6) show the expected trend; however, it may be noted that the "early-sample" advantage applies mainly to the sample in the first position, which rated highest for all foods. The individual analyses of variance showed that the over-all position effect was significant at or below the .05 level for three of the items, and an analysis (Jones and Fiske, 1953) on the combined data showed significance at the .01 level.

Results from the *contrast* design are presented in Table 4. Serving the "good" controls before the "poor" irradiated samples depressed the ratings of the later samples still further. The differences were highly significant for all four foods.

Table 4. *Contrast design.* Mean preference for control and irradiated samples.

Meat type	Control positions 1 & 2	Irradiated positions 3 & 4	Signif. of differences
Ham	7.40	5.75	.001
Pork	6.52	5.88	.02
Chicken, white	7.14	6.35	.005
Chicken, dark	6.71	5.81	.005
Av.	6.94	5.95	.001[a]

[a] Combined probability (Jones and Fiske, 1953).

Compare these averages with those for the *convergence* design (Table 5), where the irradiated samples were always served first. Again, the expected effect is demonstrated. All differences are small, but with three of the meats, the trend favors the irradiated samples. Apparently, the "poor" samples adversely affected the "good" ones.

In these two designs, contrast and convergence effects are of necessity confounded with position effect. Other analyses were made in the attempt to estimate the contribution of position *per se.* The averages for the samples in the 3rd and 4th positions of the *all-irradiated* design (Table 3) were compared with the averages for the irradiated samples in the *contrast* design (Table 4), which also were served in the 3rd and 4th positions. Results are given in Table 7. For all four foods, the *contrast* design produced lower ratings. Two-

Table 5. *Convergence* design. Mean preference for control and irradiated samples.

Meat type	Irradiated positions 1 & 2	Control positions 3 & 4	Signif. of differences
Ham	6.85	6.60	n.s.
Pork	5.96	5.92	n.s.
Chicken white	6.90	7.04	n.s.
Chicken, dark	5.90	5.83	n.s.
Av.	6.40	6.35	n.s.

Table 6. Mean preference for each serving position for combined data from *conventional, all control,* and *all irradiated* designs.

Meat type	Serving position				Signif. of linear effect
	1	2	3	4	
Ham	7.43	7.17	6.80	6.88	.01
Pork	6.46	6.22	6.46	6.52	n.s.
Chicken, white	7.22	7.18	7.00	7.03	n.s.
Chicken, dark	6.82	6.36	6.64	6.22	.05
Av.	6.98	6.73	6.72	6.66	.01 [a]

[a] Analysis of variance.

tailed *t*-tests (Jones and Fiske, 1953) for each food showed that the difference was significant for only one food; however, an analysis of the combined data showed that the over-all difference of 0.75 scale point was significant at the .01 level. Table 8 presents analogous data bearing on the convergence effect. Averages for the control samples served in the 3rd and 4th positions in the *all-control* design (Table 2) were always higher than averages for the same samples in the same positions of the *convergence* design (Table 5). The differences for ham and pork were significant at the .05 level, and the analysis of the combined data showed that the over-all difference of 0.58 scale point was significant at the .01 level. These analyses indicate that both contrast and convergence effects are more than just the incidental results of order of serving.

Table 7. Comparison of mean preferences for irradiated samples served in the 3rd and 4th positions in the *all-irradiated* and *contrast* designs.

Meat type	All irradiated design	Contrast design	Signif. of differences
Ham	6.90	5.75	.03
Pork	6.35	5.88	n.s.
Chicken, white	6.96	6.35	n.s.
Chicken, dark	6.58	5.81	n.s.
Av.	6.70	5.95	.01 [a]

[a] Combined probability (Jones and Fiske, 1953).

Table 8. Comparison of mean preferences for control samples served in the 3rd and 4th positions in the *all control* and *convergence* designs.

Meat type	All control design	Convergence design	Signif. of differences
Ham	7.29	6.60	.05
Pork	6.83	5.92	.05
Chicken, white	7.10	7.04	n.s
Chicken, dark	6.52	5.83	n.s.
Av.	6.93	6.35	.01 [a]

[a] Combined probability (Jones and Fiske, 1953).

DISCUSSION

Position effects were demonstrated in these experiments: however, they were weak. They did not appear uniformly across the four positions, but were confined almost exclusively to the change in rating from the first to the second positions. This is consistent with observations, some casual and some controlled (unpublished data), made in the Institute laboratory over a period of many years. Position effect is ubiquitous. Usually its presence can be detected, but it is highly variable. It may start early or late in the series, and it may be large or small. It seems to be affected by various aspects of the experimental situation, including such things as food type and the number of samples to be tested; and certainly we may assume that the test subjects' attitudes and expectations are involved. Bradley *et al.* (1954) reported a study that involved presentation of a series of 8–12 samples to each subject, with no position effects found. This may have been due to the subjects' consciously or unconsciously guarding against changes in their criteria in anticipation of the lengthy series.

Contrast effect was clearly demonstrated—the usual finding. Also, it was shown to be much larger, and independent of, position effect, although the two always operate in the same direction. This suggests that the contrast design might be deliberately employed to improve discrimination when testing a unidirectional hypothesis; however, because of the confounding with position effect, this should be done only when there is assurance that position effect will be negli-

gible. Seldom does one have such assurance *a priori.*

Perhaps the most important result of these experiments was demonstration of the convergence effect. Even though Kamenetzky (1959) predicted it on the basis of theory, and named it, his published data failed to show it. A major assumption in Kamenetzky's experiment was that the more preferred sample also had undesirable qualities, an assumption that perhaps was not met by foods he used (e.g., beverage bases). With meats, however, this assumption may be more reasonable. For example, Henick (1962) stated the viewpoint of some researchers that the flavor of irradiated meat results from reactions of the meat protein that, in effect, accentuate certain normal meat attributes. This implies that both the control and irradiated samples have undesirable qualities, though at different levels of intensity. Hence, the necessary conditions for the testing of Kamenetzky's hypothesis appear to have been met in this study. Here it was not only demonstrated, but, like contrast effect, was shown to be independent of position effect.

The convergence design, unlike the contrast design, would seem to have little possibility of useful application. Tests are, almost without exception, designed to detect differences under a given set of conditions. It is permissible to define conditions so as to maximize discrimination, but there is little need or justification for a design that will minimize discrimination.

A final word should be said in recognition and defense of the conventional design, where each sample is served in each position with the same frequency and equally often precedes and follows each other sample. This design is conventional because it is simply good experimental practice. Position, contrast, and convergence are real, and may affect the rating of any sample at any time. They cannot be eliminated, but their effects can be neutralized.

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