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CONTENTS

The Carotenoids of Several Low-Carotenoid Fruits

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(Manuscript received November 11, 1963)

SUMMARY

The carotenoids were examined in several fruits of low carotenoid content. Fruits studied in detail were black figs, cranberries, and Thompson seedless grapes; the examination was less comprehensive for blackberries, blueberries, strawberries, and pomegranates. The carotenoid mixtures of the various fruits were similar to those in green leaves, but (except in the grapes) the beta-carotene content was much lower. Lutein-5,6-epoxide was a major carotenoid of cranberries, and violaxanthin was the most abundant carotenoid of black figs.

In recent years this laboratory has reported the compositions of the carotenoid mixtures of a number of fruits in which carotenoids are mainly responsible for the color, including navel oranges (Curl, 1961b), tangerines (Curl and Bailey, 1957), Japanese persimmons (Curl and Bailey, 1960), cling peaches (Curl, 1959), apricots (Curl, 1960a), and tomatoes (Curl, 1961a). The investigation is now extended to seven fruits in which the color is due primarily to anthocyanins or chlorophyll: commercial American cranberries *(Vaccinium macrocarpon)*, black Mission figs *(Ficus carica)*, Thompson seedless grapes *(Vitis vinifera)*, Himalaya blackberries *(Rubus procerus)*, California blueberries (*Vaccinium* sp.), strawberries *(Fragaria chiloensis)*, and pomegranates *(Punica granatum)*. Very little was known about the carotenoids of the latter group except that in most instances the provitamin A or beta-carotene value was much lower than in the fruits in the first group.

EXPERIMENTAL

The fruits (Table 1) were obtained at local markets; in batches of 700–1000 g they were blended with 50% methanol (2 L per kg) containing magnesium carbonate. Celite 503 (10% of the weight of the fruit) was added and the mixture filtered on a Buchner funnel precoated with Celite. The filter cake was worked up as previously described (Curl, 1961a), including saponification.

The main solvent systems used in countercurrent distribution were previously described (Curl, 1953; Curl, 1960b); systems IVA and IVB are minor modifications of system IV (Table 3). Chromatography was carried out on columns of magnesia (Sea Sorb 43), 14 by *ca.* 90 mm, with a graded series of eluents (Curl and Bailey, 1959). Spectral data were obtained with a Beckman DK-2 recording spectrophotometer.

Table 1. Fruit extracted.

	Month obtained	Weight extracted (g)	Weight of $MgCO_3$ added (g per kg)
Figs	Oct.	2000	3.9
Blackberries	July	3200	20
Cranberries	Oct.	2000	35
Blueberries	Aug.	1000	16
Grapes	Sept.	2000	10
Strawberries	May	700	25
Pomegranates	Nov.	1500	23

RESULTS AND DISCUSSION

Table 2 gives the total carotenoid contents of the 7 fruits studied, and comparative data for 6 orange and red fruits previously investigated.

Countercurrent distribution. Solvent system I was used to separate the carotenoid mixtures into (I) hydrocarbons, (II) monols, and (III) diols and polyols (Table 2). Data on green bell peppers (Curl, unpubl.), 2 species of leaves, and 6 high-carotenoid orange and red fruits are presented in Table 2 for comparison. In the 7 low-carotenoid fruits, the diol-polyol fraction predominated, and the monol fraction was small. The compositions resembled that found in green bell

Table 2. Countercurrent distribution with solvent system I [a] of carotenoid mixtures from fruits and leaves.

	Total carotenoids mg/kg [b]	Percentage composition		
		I [c]	II	III
Figs	8.5	11.3	2.1	86.5
Blackberries	5.9	12.2	3.2	84.7
Cranberries	5.8	7.9	4.0	88.1
Blueberries	2.7	11.0	2.2	86.7
Grapes	1.8	31.5	2.1	66.3
Strawberries	0.64	14.1	1.5	84.3
Pomegranates	0.16	15.1	5.1	79.8
Green bell peppers	10.6	13.5	1.3	85.1
Valencia orange leaves	215	25.6	1.7	72.7
Pyracantha leaves	163	35.9	2.0	62.1
Navel orange pulp	23	1.9	16.0	82.2
Tangerine pulp	27	8.1	40.2	51.7
Japanese persimmons	54	16.2	39.5	44.3
Cling peaches	27	16.9	17.2[d]	66.1
Apricots	35	88.0	6.3[e]	5.7
Tomatoes	51	93.9	0.9	5.2

[a] Hexane–99% methanol.
[b] As beta-carotene.
[c] Fraction I, hydrocarbons; II, monols; III, diols and polyols.
[d] Includes 1.0% monol epoxides.
[e] Includes 0.8% monol epoxides.

peppers, excepting that the grape carotenoids were more like those in green leaves. The 6 high-carotenoid fruits were different in that the hydrocarbon fraction predominated in apricots and tomatoes, and in the others the monol fraction was much greater than in the low-carotenoid fruits.

The diol-polyol fractions from the low-carotenoid fruits (except strawberries) were further fractionated by countercurrent distribution with solvent system IV, IVB, or II (Table 3). Diols predominated except in figs, in which the largest fraction was diepoxide diols. In cranberries and blueberries, the monoepoxide diol fraction (IIIB) was much higher than in the other low-carotenoid fruits, green peppers, and leaves; both of these fruits are *Vaccinium* species. Throughout Table 3, the diepoxide diol fraction (IIIC) is greater than the polyol fraction (IV): in most cases, including green peppers and leaves and all of the low-carotenoid fruits except figs, the ratio of IIIC to IV is around 1.6 to 1. In figs and several of the high-carotenoid fruits, this ratio is much higher, and probably indicates that in these fruits there is a considerable increase in diepoxide diols on ripening. The polyol fractions were uniformly rather low. In no fruit studied so far did the polyol carotenoid fraction exceed 15% of the total carotenoids; in many cases it was much less. The results for the low-carotenoid fruits are in general agreement with those for green peppers and leaves, while the 6 high-carotenoid fruits were quite different.

Table 3. Countercurrent distribution of diol-polyol fraction from fruits and leaves.

	Solvent system [a]	Percentage of total carotenoids			
		IIIA [b]	IIIB	IIIC	IV
Figs	IV	30.4	2.0	45.6	9.5
Blackberries	IV	46.6	1.9	22.0	14.1
Cranberries	IV	45.7	15.1	19.4	7.9
Blueberries	IVB	42.2	13.7	19.4	11.4
Grapes	IV	28.4	6.0	19.9	12.2
Pomegranates	II	39.9	.....	25.6	14.3
Green bell peppers	IVB	42.9	0.5	26.5	15.3
Valencia orange leaves	II	45.5		16.3	10.9
Navel orange pulp	II	5.2	17.8	54.1	4.9
Tangerine pulp	II	9.3	17.0	21.9	3.6
Japanese persimmons	IVA	21.8	13.6	4.9	3.9
Cling peaches	II	7.8	8.5	42.5	7.3
Apricots	II	3.5	6.3	8.8	6.3
Tomatoes	IV	2.9	0.3	1.4	0.7

[a] Solvent systems: IV, hexane, 73.5% methanol; IVA, hexane, 75% methanol; IVB, hexane, 70% methanol; II, hexane, benzene, 87% methanol (1:1:1.15).
[b] Fraction IIIA, diols; IIIB, monoepoxide diols; IIIC, diepoxide diols; IV, polyols.

Countercurrent distribution runs with solvent system II provided larger quantities of material for chromatography. Aliquots of the diol-polyol fraction (III) separated with solvent system I from figs, cranberries, and grapes, and aliquots of the total carotenoids from blackberries and blueberries were used.

Chromatography. Chromatography of all strawberry and pomegranate fractions, and of some fractions from other fruits, was omitted because of their low carotenoid content. All fractions from cranberries were chromatographed, resulting in the isolation of 23 components, many more than from the other fruits (Table 4). The spectral absorption maxima for the cranberry fractions are given in Table 4; the values obtained for the corresponding constituents of the other fruits did not differ significantly from these.

beta-Carotene was the most abundant constituent of the hydrocarbon fraction in all of the fruits; in grapes it was the major constituent of the total carotenoids. The alpha-carotene band from grapes had maxima also

Table 4. Carotenoid constituents obtained from low-carotenoid fruits by chromatography.

Constituent	Spectral absorption maxima[a] (mμ) (cranberries)	Approximate percentage of carotenoid mixture[b]				
		Cranberry	Fig	Grape	Blackberry	Blueberry
Hydrocarbons (I)						(11.0)[c]
Phytoene	(297),[d] 285,275	4.7	2.4	+	1.5	
Phytofluene	366,348,330	0.9	0.7	0.2	0.2	
alpha-Carotene	471,441,418	0.14	0.24	0.2	1.1	
Unidentified	396,378,(356)[e]	....	....	0.4	....	
beta-Carotene	475,448,(424),335	5.3	9.9	32.3	9.5	
zeta-Carotene	422,398,376	0.6	....	....	....	
Mutatochrome[f]	450,425,(404)	0.6	....	....	....	
Monols (II)			(2.1)[c]	(2.1)[c]	(3.2)[c]	(2.2)[c]
Hydroxy-alpha-carotene-like	469,440,414	0.4				
Hydroxy-alpha-carotene	471,440,416,334	1.1				
Cryptoxanthin	474,445,(422),335	1.8				
Diols (IIIA)						
Lutein	485,453,430,338	31.3	28.8	22.0	44.3	39.2
Zeaxanthin	488,459,(433),345	2.8	1.1	1.1	2.3	2.1
Monoepoxide diols (IIIB)			(2.0)[c]	(6.0)[c]	(1.9)[c]	(13.7)[c]
Carbonyl[f]	(443),417	0.18				
Lutein-5,6-epoxide	479,448,422,335,321	10.5				
Antheraxanthin (cis)	479,453,427,337	4.6				
Lutein-5,8-epoxide	457,428,404,317	0.7				
Mutatoxanthins	462,434,413,319	0.33				
Diepoxide diols (IIIC)					(22.0)[c]	(19.4)[c]
Carbonyl[f]	(432),406	0.37	....	....		
Violaxanthin	479,447,422,335,321	20.7	40.0	15.7		
Luteoxanthins	459,430,405,318,303	1.8	2.5	1.6		
Polyols (IV)					(14.1)[c]	(11.4)[c]
Valenciaxanthin[f]	399,376,359	1.1	0.4			
Sinensiaxanthin[f]	427,402,380	0.25	1.0			
Neoxanthin	477,447,421,335,322	6.9	7.1	(12.2)[g]		
Sinensiaxanthin-like	428,404,382	1.8	1.0			

[a] Fractions I and II in hexane, others in benzene.
[b] Percentages based upon total absorbance of each constituent at its principal spectral absorption maximum.
[c] Percentage of fraction from countercurrent distribution; fractions not chromatographed.
[d] Values in parentheses indicate inflections or humps on spectral absorption curves.
[e] Data for grape fraction.
[f] Tentative identification.
[g] Spectral absorption curve of entire fraction very similar to that of neoxanthin.

at 396 and 378 mμ, with a shoulder at 356, indicating the presence of an unidentified constituent in about twice the amount of the alpha-carotene.

The monol fraction was minor in all fruits and was not examined chromatographically except in cranberries. A spectral absorption curve was run on the entire fraction from grapes; the maxima, in hexane, were at 471, 443, 418, 397, and 373 mμ, indicating a mixture to be present, probably including hydroxy-alpha-carotene.

The preponderance of lutein to zeaxanthin in the diol fraction was not characteristic of most of the high-carotenoid fruits in which there is, apparently, an increase in zeaxanthin on ripening.

The amount of lutein-5,6-epoxide in cranberries was greater than that of beta-carotene and neoxanthin, and was exceeded only by lutein and violaxanthin. Lutein-5,6-epoxide had been found previously in only a few fruits, where it occurred as a very minor component.

The spectral absorption curve of lutein-5,6-epoxide is very similar to that of violaxanthin (zeaxanthin-5,6,5′,6′-diepoxide), which has the same conjugated double bond system. The N_{100} value in solvent system II was 57, in good agreement with the value of 55 for antheraxanthin (zeaxanthin-5,6-epoxide) and much higher than the value of 40 for violaxanthin. In ethanol solution, the spectral absorption maxima changed from 467, 436, and 413 mμ to 447, 419, and 396 on addition of one drop of concentrated hydrochloric acid to the cuvette, indicating the original substance to be a 5,6-monoepoxide.

A test was made for allylic hydroxyl groups by treating lutein-5,6-epoxide with hydrochloric acid in methanol (Curl, 1956). Countercurrent distribution with solvent system I showed the N_{100} value of the main product to be 33; the corresponding value of lutein in this system was 10. The expected value for lutein-5,6-epoxide is about 8. Lutein, however, has an allylic hydroxyl group, and, on treatment with hydrochloric acid in methanol, it forms a monomethyl ether with N_{100} value in system I of 39. The lower N_{100} value of the main product is attributed to the presence of the 5,8-epoxide group in lutein-5,8-epoxide-3′-methyl ether.

The N_{100} value of 33 for the substance identified as lutein-5,6-epoxide-3′-methyl ether is in close agreement with the values of 32-33 and 31 in the same solvent system for, respectively, cryptoxanthin 5,6,5′,6′-diepoxide and the corresponding 5,8,5′,8′-diepoxide (Curl, 1962). The effect of an epoxide group (either 5,6- or 5,8-) and of a 3- or 3′-methoxy group is thus about the same, on countercurrent distribution with system I.

In cranberries, figs, and grapes, the principal constituent of the diepoxide diol fraction was identified as violaxanthin. In the cranberry carotenoids, a minor fraction with spectral absorption maximum in benzene at 406 mμ, with a shoulder at 432, is apparently a carbonyl carotenoid; a similar substance with somewhat higher-wavelength spectral absorption maximum was found in the monoepoxide diol fraction.

Because of the small amount of material available, the polyol fraction from Thompson grapes was not chromatographed, but a spectral absorption curve was run directly. The curve, with maxima in benzene at 476, 444, 419, 335, and 317 mμ, corresponded closely to that of neoxanthin, which was the major component of this fraction in both the fig and cranberry carotenoids. Two minor constituents from both figs and cranberries, which occurred below neoxanthin on the column, were apparently valenciaxanthin and sinensiaxanthin, 5,6-epoxides of incompletely known structure originally found in Valencia orange juice (Curl and Bailey, 1954). Above neoxanthin on the column was a substance with spectral absorption maxima similar to those of sinensiaxanthin. Unlike the other 3 substances found in this fraction the spectral absorption maxima of this substance showed no significant change on addition of hydrochloric acid to solutions in ethanol, showing that it was not a 5,6-epoxide.

Of the low-carotenoid fruits investigated, Thompson seedless grapes most resembled green leaves in carotenoid compositions; the others had a considerably lower hydrocarbon carotenoid content (principally beta-carotene). The cranberry carotenoids also differed from leaf carotenoids in having lutein-

5,6-epoxide as a major carotenoid. A rather high content of violaxanthin in the fig carotenoids may represent a moderate increase resulting from ripening; the high ratio of diepoxide diols to polyols also indicates this. Otherwise, in the seven low-carotenoid fruits studied, there seems to have been little or no increase in the carotenoids on ripening; the carotenoids present appear to be essentially the residue of those present in the green fruit.

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Volatiles and the Harvest Maturity of Peaches and Nectarines[a]

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SUMMARY

Volatiles emanating from ripening fruit harvested at different stages of maturity were analyzed with direct sampling techniques and a flame-ionization-equipped gas chromatograph. All fruits within the selected maturity range ripened into commercially salable products. Although firmness, soluble solids, and carbon dioxide production indicated a nearly equivalent ripening of the different maturity categories the presence of detectable volatiles was markedly dependent upon the maturity at harvest. Additional volatile fractions were detected when the fruit was cut prior to sampling. Possible use of these techniques in fruit maturity evaluations is discussed.

INTRODUCTION

Many of the physicochemical properties of maturing peaches have been studied in order to obtain a suitable indicator of harvest maturity (Allen, 1932; Haller, 1952; Romani *et al.*, 1962). The need remains, however, for an objective index of fruit maturity, and especially for an index that correlates well with organoleptic acceptance of the product. Morris (1932) first alluded to the importance of volatiles in judging fruit maturity when he reported that the aroma increased slightly as fruit softened, regardless of picking maturity, but that less mature fruits never attained the prized fragrance of those harvested in a more mature state.

In early experiments, Power and Chesnut (1921) used laborious chemical methods to isolate essential oils from peaches. Later, Daghetta *et al.* (1956) used similar techniques to isolate odorous compounds evolved during the storage of fruit. Studies of fruit volatiles have been few, for want of a simple and sensitive method of analysis, a lack that was corrected by the advent of gas chromatography and the flame ionization detector (McWilliams and Dewar, 1958).

A ripened fruit that is palatable and acceptable to the consumer either as a fresh or a processed product reflects an adequate maturity at harvest. Since volatiles are intimately associated with palatability, they could well serve as tangible evidence of fruit maturity. The data presented herein were derived, not from a study of flavor components *per se*, but rather from an attempt to use direct gas chromatographic analysis as a tool to investigate the relationship between the emanation of volatiles and the harvest maturity of fruit.

MATERIALS AND METHODS

Two varieties of peaches, Red Globe and Elberta, and Early Sun Grand (ESG) nectarines were obtained at the time of commercial harvest in orchards near Fresno, California. Each variety was separated into three maturity categories: more mature (M3), intermediate maturity (M2), and the least mature fruit (M1) that was being picked for commercial shipment (although in some cases when the spread in maturity was not as great, somewhat greener fruits were picked and designated as M1). The freshly picked fruits were first cooled, and then transported to Davis. (As used herein, the term "mature" refers to the physiological state at time of harvest, and the term "ripe" refers to the dessert condition of the fruit after ripening, i.e. at the end of the experiment.)

Firmness and soluble solids of eight fruits from each experimental group were measured with a pressure tester (5/16-inch tip) and a hand refractometer at both the beginning and end of the experiment. Eight to ten fruits were placed in a gallon jar, which was then sealed except for inlet and outlet tubes. Four jars of fruit at each maturity stage were prepared as replicates. An

[a] Presented at the Western Regional meeting of the AAAS, June, 1963.

empty jar was used as a control to account for the presence of volatiles in the air or for those that might arise from the rubber jar tops or tubing. The jars were placed at 68±1°F, and air, saturated with water vapor to prevent fruit dehydration, was passed through each jar at a flow rate of 350–400 ml per minute. Carbon dioxide production was measured by the Claypool-Keefer technique (1942) and calculated as mg CO_2/hr/kg fruit. Extra fruit was kept at the same controlled temperature for cut-fruit experiments and for the replacement of experimental fruit showing inception of a mold.

Gas sampling techniques. To allow for daily chromatographic analysis, each jar of fruit was taken off the air line at appropriate intervals and immediately sealed by interconnecting the inlet and outlet tubes. After 30 min the needle of a Cornwall Luer-Lok automatic syringe was inserted through the rubber top of the jar, and the syringe was filled and emptied several times to ensure complete mixing of the air about the fruit. After the final withdrawal, the 5-ml air sample was injected into the gas chromatograph. Immediately after sampling, the jar was connected back onto its air line. Volatile residues in the syringe were removed by placing the syringe either under vacuum or in a warm oven. In the latter case the syringe was cooled to room temperature before reuse. Some aspects of this procedure correspond to the "aromagram" method of Buttery and Teranishi (1961).

In experiments designed to measure the emanation of volatiles from cut fruits, six fruits from each maturity group were first halved, and 200 g of tissue was then taken from each of the six halves and diced as rapidly as possible. The diced fruit was then put into a 300-ml Erlenmeyer flask and covered snugly with aluminum foil. After 30 min, a 5-ml sample of air was withdrawn from the flask and injected into the gas chromatograph. A duplicate determination was made on the remaining fruit halves. Nearly similar procedures were being used concurrently by Ponting (1963) to measure the volatiles of cut pears.

Chromatographic conditions. A Loenco gas chromatograph with a hydrogen flame detector was used. After preliminary tests on three early varieties of peaches, the following analytical conditions were established:

Column packing: 8% diethylene glycol succinate (DEGS) on acid-washed chromosorb P (30/60-mesh)

Column size: ¼-inch × 10 feet

Oven temperature: 95±2°C for peaches and 115±1°C for nectarines

Injector temperature: 150°C (achieved by wrapping a thermal tape around injector)

Recorder: 1-mv full-scale Minneapolis-Honeywell recorder

Chart speed: ¼-inch/min

Carrier gas and flow rate: nitrogen at 12.5 ml/min

Hydrogen gas flow rate: 20 ml/min

Air flow rate: 300 ml/min

All the flow rates were measured separately at the outlet of the burner and at room temperature.

All volatile components of the injected samples would elute off the column within 40 min. Retention times were used for peak designation. The areas of the chromatographic peaks were measured according to the method of approximating triangles (Pecsok, 1959) and expressed in sq cm per kg fruit to facilitate quantitative comparisons.

Ethylene identification. During several preliminary gas chromatographic analyses, a compound with a very short retention time was repeatedly the first detectable volatile produced by ripening peaches. An appropriate concentration of ethylene gas cochromatographed with the fruit volatiles had the same retention time as the low-boiling unknown. Corroborative evidence was obtained by passing the gas sample from the fruit jar through a mercuric perchlorate solution that Young *et al.* (1952) showed absorbs ethylene selectively. This was done by bending the lower capillary portion of a disposable pipette into a "J" and inserting it through a small hole made in the bottom of a test tube, which was then partially immersed in a solution containing 0.25*M* mercuric perchlorate in 2*M* perchloric acid. The ends of both pipette and test tube extending above the solution were fitted with rubber caps. A 5-ml sample of gas was injected slowly into the "J" pipette and collected in the upper portion of the test tube. No ethylene was detected if a diluted ethylene sample was first passed through the mercuric perchlorate solution. Similarly, the ethylene peak was completely eliminated from the gas sample taken from a jar of fruit when the gas was first passed through the mercuric perchlorate solution several times.

RESULTS

Physiological state of the fruit. To affirm that the changes in volatile production were indeed those that accompanied the ripening process, soluble solids and firmness of peaches and nectarines were taken before and after completion of the experiment (Table 1). The firmness values afford an indication that the maturity difference between M1 and M3 fruit was no greater than that often encountered in the product harvested for commercial use. In general, there was a slight increase in soluble solids content during the ripening process. All of the samples in both the M1 and M3

Table 1. Soluble solids and firmness data for peaches and nectarines.

Variety of fruit	Picking maturity	Soluble Solids (%) At harvest	Soluble Solids (%) After ripening	Firmness (lb) At harvest	Firmness (lb) After ripening
Red Globe peach	M1	11.7	13.4	15.1	1.0
	M3	12.9	10.9	7.5	0.7
Elberta peach	M1	12.5	13.0	14.6	1.1
	M3	13.5	14.0	4.7	0.5
Early Sun Grand nectarine	M1	9.1	9.2	9.7	1.0
	M3	12.2	12.5	4.2	0.5

categories softened and developed into salable, ripe fruit.

Chromatographic results. Fig. 1 shows a typical trace obtained with volatiles from M3 Red Globe peaches. To facilitate a comparison of the various chromatographs, a schematic representation was made wherein each peak is identified by its retention sequence. In addition, the base of the peak is drawn at a fixed width, or twice this width for exceptionally large peaks, and the height adjusted to represent the actual peak area. For ease of reference a consecutive number was given to each successive fraction following the ethylene peak. Thereafter, volatile fractions with the same retention time were assigned the same number.

Fig. 2 shows chromatograms of the volatiles produced by M1 and M3 Elberta peaches and ESG nectarines. Qualitatively and quantitatively, more volatiles are produced by the ripening M3 fruit than by the M1. This is in contrast to the lack or near lack of volatiles from either the M1 or M3 categories at the time of harvest.

Table 2 summarizes the presence or absence of each volatile component for each variety. Since Red Globe peaches produced the most detectable volatile fractions, these were used for numerical reference in order of elution, as indicated above. However, since the chromatograph oven temperature was higher for nectarines than for peaches, a similar numerical designation does not imply the

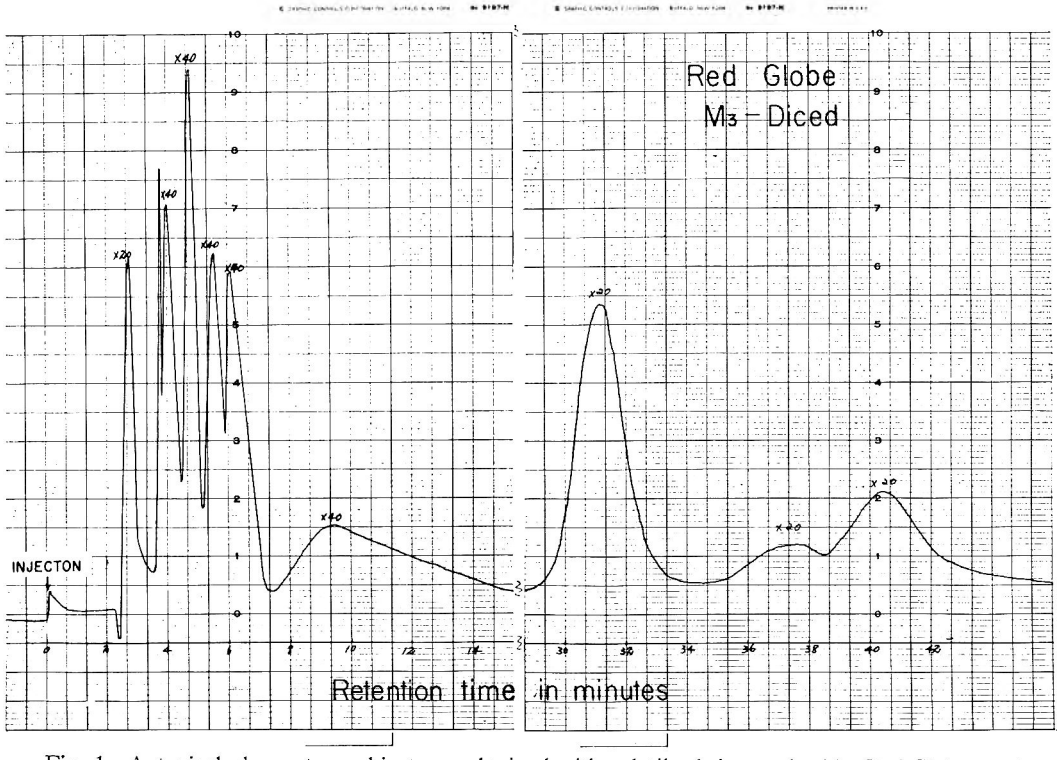

Fig. 1. A typical chromatographic trace obtained with volatiles being evolved by Red Globe peaches. The last three fractions (peaks 6, 7, and 8) were detected only with diced fruits.

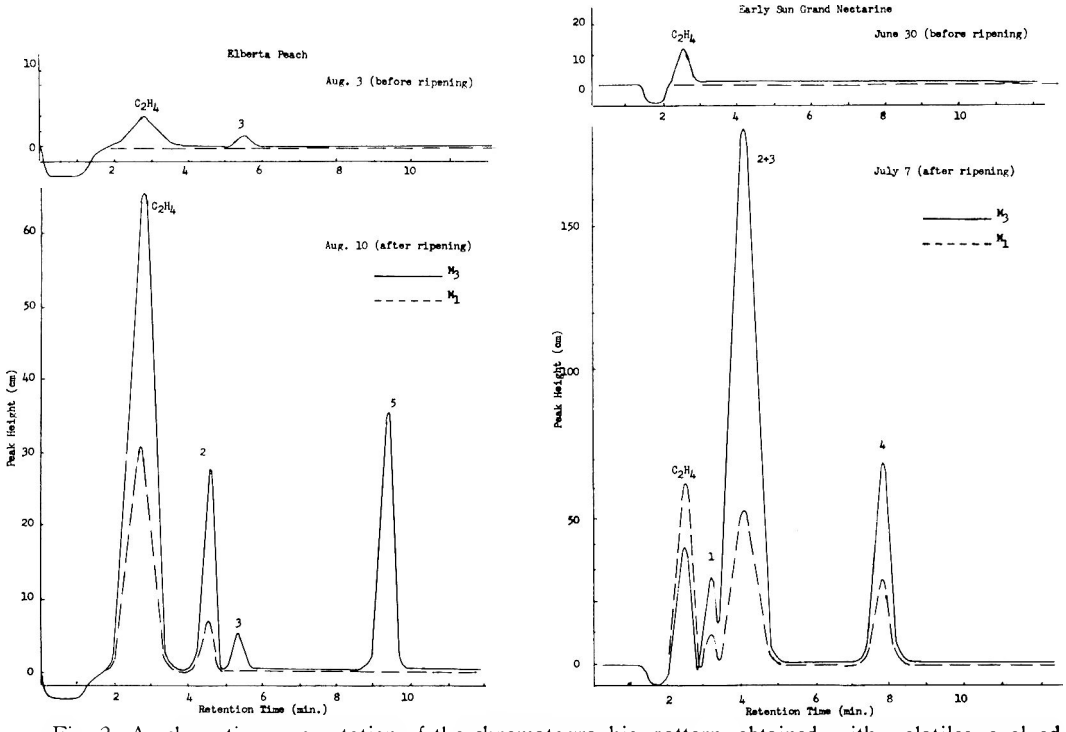

Fig. 2. A schematic representation of the chromatographic pattern obtained with volatiles evolved from Elberta peaches and Early Sun Grand nectarines before and after the ripening period. More mature (M3) and less mature (M1).

Table 2. Designation of chromatographic peaks corresponding to peach volatiles (ethylene peak excluded).

Peach varieties		Peak no. 1	2	3	4	5
Elberta	M1	—	+	—	—	—
	M3	—	+	+	—	+
Red Globe	M1	—	+	—	—	+
	M3	+	+	+	+	+

+ present; — absent.

same volatile. Table 3 shows the retention times for all chromatographic peaks.

Time course for volatiles production. Fig. 3 shows the appearance of volatile fractions and the time course for their production. Each point on the graphs represents the averaged peak area from separate chromatograms of the volatiles from each of the four replicate jars. The ethylene peak is not included in these or subsequent data.

Both the number of volatile fractions and the rate of their production by M3 Elberta are in marked contrast to the single volatile fraction, No. 2, produced by fruit of the same variety but harvested when less mature. Volatile fraction 5 was not evolved by the more mature Elberta peaches until the third day of ripening. A delay in the production of this volatile fraction was also indicated by the Red Globe peaches, its presence being detectable on the third day of ripening for the M3 and the sixth day for the M1 category. Additional fractions 1 and 4 were also evolved by the more mature Red Globes. The erratic production rate of volatile fraction 3 by the Red Globe peaches is difficult to explain. The period

Table 3. Retention times for the volatile fractions obtained from peaches and nectarines.[a]

Peaches[b] Fraction no.	Retention time min	sec	Nectarines[b] Fraction no.	Retention time min	sec
C_2H_4	2	43	C_2H_4	2	34
1	4	1	1	3	16
2	4	36	2	3	44
3	5	26	3	4	8
4	6	1	4	7	52
5	9	27			
6 cut fruit	31	6			
7 cut fruit	37	19			
8 cut fruit	39	50			

[a] Chromatographic conditions as given in the text.

[b] Respective temperatures of chromatograph oven were 95 and 115°C for the analyses of peach and nectarine volatiles.

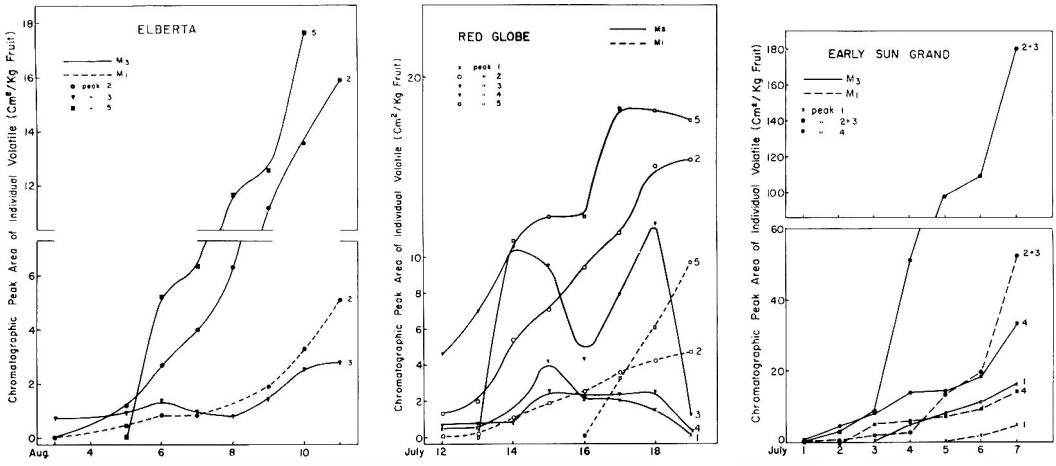

Fig. 3. The rate of production of individual volatile fractions by Elberta and Red Globe peaches, and Early Sun Grand nectarines during their ripening process.

of diminished production of the volatile fraction did correspond to the replacement of some fruit that had shown the first signs of mold; however, a similar but less pronounced drop in the production of this volatile was indicated for the Elbertas, where no fruit had been replaced.

Volatile fractions 2 and 3 were not adequately resolved from the nectarine emanations to be plotted separately. The combined area of the chromatographic peaks for these two fractions increased severalfold during the ripening process, the rate of increase being considerably greater for the more mature fruit. Other volatile fractions exhibited similar trends corresponding with maturity differences.

A comparison of total volatiles and CO_2 production. Assuming a general contribution of volatiles to fruit flavor, their total effect may be represented by a summation of the volatile fractions detected at a given sampling. Ethylene was excluded from this summation in view of its recognized special physiological role (Biale *et al.*, 1954) and doubtful direct contribution to flavor.

Total volatiles evolved by ripening Elberta peaches and ESG nectarines (Fig. 4) indicate a marked difference between the M1 and M3 fruit.

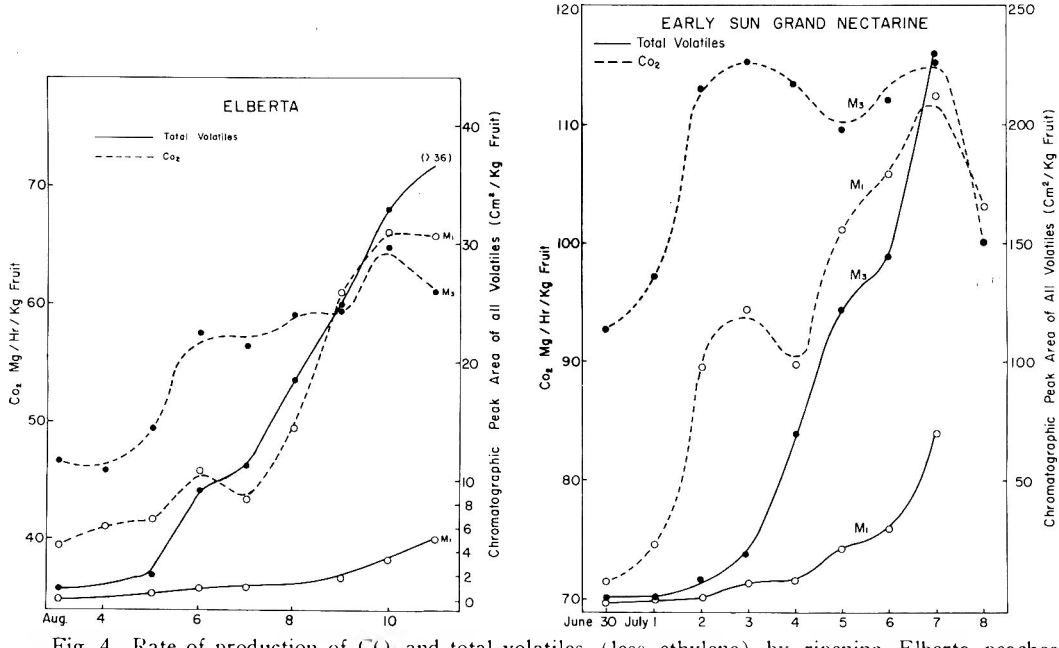

Fig. 4. Rate of production of CO_2 and total volatiles (less ethylene) by ripening Elberta peaches and Early Sun Grand nectarines.

At the ripe stage, the more mature harvested peaches yielded *ca.* six times as much volatiles, and the more mature nectarines *ca.* three times as much volatiles, as comparable but less mature fruit. This disparity occurred in spite of the fact that the fruit in both maturities softened and generally achieved a similar state of ripeness as indicated by the terminal respiration rates and coincidence of the climacteric peaks. It was also noted that nectarines respired at nearly double the rate of peaches, corresponding to similar findings by L. L. Claypool (personal communication).

Volatiles emanating from cut fruit. Chromatograms of volatiles from cut fruit are represented by the results with Red Globe peaches (Fig. 5). Before ripening, the cut M1 fruit evolved only 5 identifiable volatile fractions, 2 less than the 7 fractions for the M3. These are in addition to the ethylene fraction. Of the volatile fractions that correspond to those also detected in intact fruit

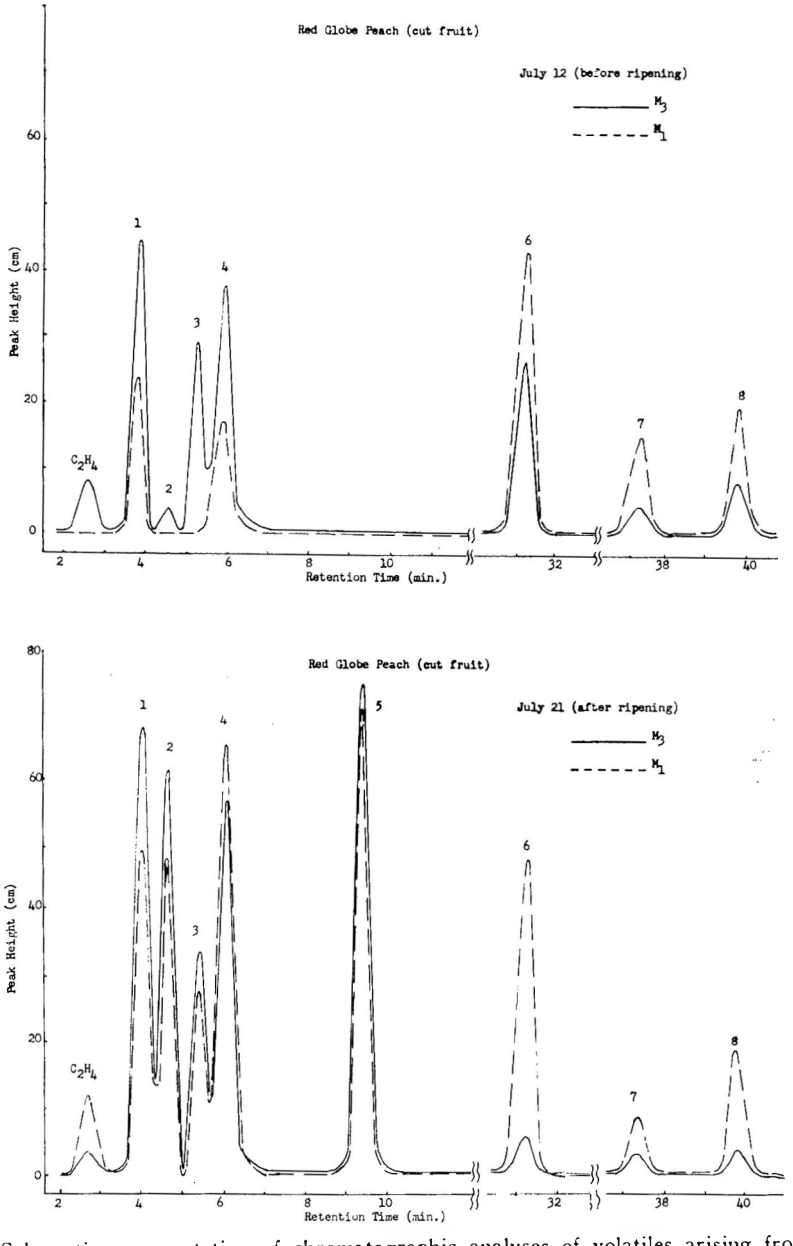

Fig. 5. Schematic representation of chromatographic analyses of volatiles arising from diced Red Globe peaches.

(Nos. 1–5) larger amounts were evolved by the riper cut fruits. However, fractions 6, 7, and 8, which were detected only with cut fruit, were produced in higher amounts by the less mature fruit. This suggested that the volatile substances represented by fractions 6, 7, and 8 might increase during maturation and the first portion of the ripening process, but decrease thereafter. Although not as marked, the same pattern was indicated in similar experiments with Elberta peaches.

DISCUSSION

At the end of the ripening period both M1 and M3 fruit had nearly equivalent respiration rates and almost coincident climacteric peaks. In addition, all fruits had softened to less than 1 lb firmness, negating the possibility of a significantly longer storage period or increased volatiles production without incurring overripeness. This suggests, therefore, that the rate of volatiles production in the postharvest period is strongly dependent on the maturity at harvest.

The number of different detectable volatile fractions also increased with harvest maturity. For instance, volatile fraction 5 was evolved by M3 Elbertas after the third day of ripening but never evolved by the less mature M1 fruit (Fig. 3). Both M1 and M3 Red Globes, which were on the whole more mature than the Elbertas, evolved volatile fractions 5, but not until the sixth day of ripening for the less mature (M1) compared with the third day for the more mature (M3) category (Fig. 3).

In addition to the effect of maturity on volatiles production, different varieties of peaches may have special essences that contribute toward their characteristic flavor. For example, volatile fraction 4 was prominent in the Red Globe but was not evolved by the ripening Elberta. Far more subtle differences undoubtedly exist beyond the range of the methods employed and current instrumentation.

Different means of preparing the fruit tissues were noted to cause changes in the volatiles evolved. If the peaches were gently homogenized rather than diced, the retention time of the sixth chromatographic peak was shortened without otherwise altering the chromatographic pattern. Blending in a dilute solution of cysteine did not prevent this change in the volatile fraction. Observations of this nature suggest that classical procedures of preparing flavor essences may have markedly altered the identity of the isolated fractions.

Whereas the amount of volatiles produced by whole fruit increased with fruit ripeness, other volatile fractions, detected only with cut fruit, were present in larger amounts at an intermediate ripeness. The production of volatile fractions 6, 7, and 8, having a considerably longer retention time and detected only with cut fruit (Fig. 5), began to decrease before fruit reached its climacteric peak. The significance of this divergent rate of volatiles production in determining optimum ripeness for fruit consumption or processing should be evaluated with the use of taste panels.

This is a first attempt to rationalize fruit maturity differences in terms of their volatile constituents. The data are too limited to warrant any definite conclusions. Future research with improved chromatographic techniques, identification of the volatile fractions, and organoleptic evaluation by taste panels, would be necessary. These data do suggest, however, that chromatographic criteria may be useful in identifying adequate maturity standards for individual varieties or perhaps whole species of fruit.

ACKNOWLEDGMENT

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The Influence of Variety on the Pyrrolidonecarboxylic Acid of Canned Beets

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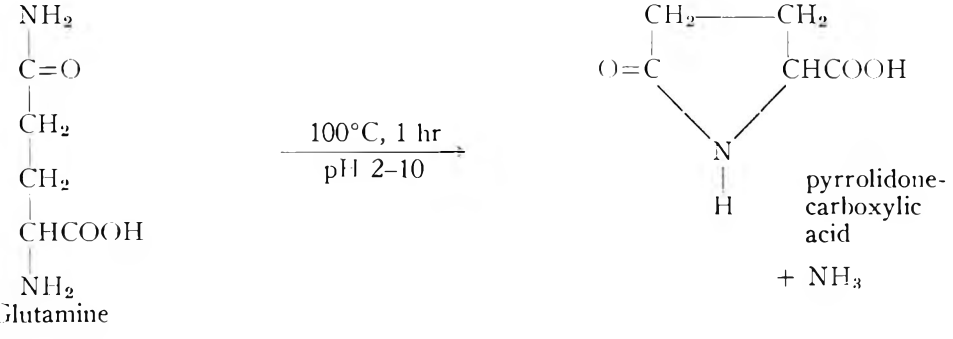
SUMMARY

The greatest amount of pyrrolidonecarboxylic acid (PCA) found in any of 10 varieties of processed beets was less than has been reported to be necessary to cause a flavor defect. The difference in PCA content between the varieties of canned diced beets containing the greatest and smallest amounts of PCA was less than reported necessary to affect flavor preference. The beets were grown on muck soils without additional nitrogen fertilizer. The relatively low level of PCA in the beets was probably due, in part at least, to their being grown on unfertilized muck soil. A significant difference in PCA content was found in certain varieties of processed beets. Pieters-Wheller Early Blood Turnip beets had significantly less PCA than Rohnert-Detroit Dark Red, Rohnert-Early Wonder Tall Top, and Robson Seneca Detroit varieties.

INTRODUCTION

Many factors are believed to have an effect on the pyrrolidonecarboxylic acid (PCA) content of canned fruits and vegetables. These affect the glutamine content of the raw product, which is directly related to the content in the canned product when the latter is processed at 240°F or above or is processed at lower temperatures and stored long enough to allow conversion of the remaining glutamine to PCA (Mahdi, 1959).



PCA may have a deleterious effect on the flavor of canned beets, especially when the levels exceed 200 mg per 100 g drained weight. Beets contain relatively large amounts of glutamine, PCA's precursor, and therefore contain correspondingly large amounts of PCA in the canned form. A study was undertaken to determine the variation in glutamine and PCA content of several varieties of beets and their suitability for canning.

METHODS

Test beets used were Rohnert-Detroit Dark Red, 1-E4-13; Robson-Seneca Detroit, 1675L; Pieters-Wheller Early Blood Turnip; Pieters-Wheller Special Crosby; Rohnert-Detroit Dark Red, 1-E4-11; Rohnert-Early Wonder Tall Top S/N 502404; Northrup-King Perfected Detroit 74/19; Rohnert-Perfected Detroit 501 304; Northrup-King Ruby Queen 74/5; and Northrup-King King Red 83/13, all grown on muck soil. Fertilizer was not used on the the plot; in the previous year the plot was fertilized with 1000 lb/acre of 10-10-10 fertilizer.

Processing of the beets was conducted in a manner similar to methods commonly used in Wisconsin canneries; steam blanching instead of lye soaking was used, followed by abrasion peeling. The beets were washed in warm water and size-graded; only raw beets 1½–3 inches in diameter were processed. The beets were steamed 15 min in a retort at 210°F, mechanically peeled in a rotary abrasion peeler, and diced into ¼-inch cubes. The diced beets (300-g portions) were packed in 300 × 407 enamel-lined cans and covered with water at about 200°F. The filled cans were exhausted for 6 min at 200°F, sealed, heat-processed 40 min at 240°F, and immediately cooled. After processing, the canned beets were stored 2 months at 86°F, and then frozen until analyzed.

The method used to measure the PCA content quantitatively involved partition chromatography on a silicic acid column as developed by Martin and Synge (1941), modified by Rice and Pederson (1954), and further modified by Mahdi (1959). Some slight further modifications were made in the method. A column, 10 mm inside diameter, was packed with 6.5 g of silicic acid [a] saturated with 3.0–3.5 ml of 0.1*N* H_2SO_4 and mixed in 20–30 ml of chloroform. The chloroform was equilibrated [b] with 0.1*N* H_2SO_4 and filtered through dry filter paper.

The solvent schedule consisted of 150 ml of A.R.-grade *n*-butanol in U.S.P. grade chloroform (V/V). These solvent mixtures were equilibrated [c] with 0.1*N* H_2SO_4 and filtered through dry filter paper just before using.

The canned beets were prepared for analysis by acidifying the beet liquor to pH 1.0–2.0 with concentrated H_2SO_4 and mixing 0.50 ml with 0.75 g of silicic acid. The sample preparations were added to the tops of the columns with the aid of a powder funnel; care was taken to prevent the column from running dry. The PCA content of the beet liquor of the canned beets was measured, and the PCA content of the beets was expressed as mg PCA per 100 g of drained weight (AOAC, 1955) using the following formula:

$$\frac{(\text{ml NaOH used})\ (\text{N of NaOH})\ (129.12\ \text{mg/meq of PCA})\ (\text{ml of liquor})}{(\text{ml of sample})\ (\text{gram of drained weight})} \times 100 =$$

mg PCA/100 g drained weight of processed beets

[a] One pound of silicic acid (Baker's A.R. grade) was washed with 5 L of distilled water and allowed to stand undisturbed for 10 min. The supernatant water containing the very fine particles of silicic acid was decanted and the washing repeated a second time. The residual silicic acid was then activated by drying, with occasional stirring, in an oven at 120°C for 18–20 hr. The silicic acid was sieved; the portion passing through a 100-mesh sieve was retained and dried an additional 4–6 hr at 120°C before using. The silicic acid thus obtained required 2–5 lb of pressure to elute 10 ml of solvent through a 10-mm-diameter column in the desired 8–10 min.

[b] All solvents were equilibrated in a 500-ml separatory funnel with about 50 ml of 0.1*N* H_2SO_4.

[c] All solvents were equilibrated in a 500-ml separatory funnel with about 50 ml of 0.1*N* H_2SO_4.

RESULTS AND DISCUSSION

Table 1 shows the PCA content of the processed beets, arranged in ascending order. The values are averages of measurements made at four maturity levels.

Table 1. Average PCA content of 10 varieties of canned diced beets.

Variety	PCA (mg per 100 g drained wt.)	Signif. diff. at 5% level
1. Pieters-Wheller Early Blood Turnip	89.6	a
2. Pieters-Wheller Special Crosby	97.9	ab
3. Northrup-King King Red 83/13	99.9	ab
4. Northrup-King Ruby Queen 74/5	105.2	ab
5. Northrup-King Perfected Detroit 74/19	106.4	ab
6. Rohnert-Perfected Detroit 501 304	106.4	ab
7. Rohnert-Detroit Dark Red 1-E4-13	108.3	ab
8. Rohnert-Early Wonder Tall Top S/N 502 404	110.1	b
9. Rohnert-Detroit Dark Red 1-E4-11	111.9	b
10. Robson-Seneca Detroit 1675 L	113.1	b

The difference between the greatest and least amounts of PCA in the ten varieties of canned beets was 23.5 mg per 100 g of drained weight, and the range 89.6–113.1. Duncan's new multiple-range test at the 5% level was applied to the data; the results are shown in Table 1.

Any two means not identified by the same letter differ significantly at the 5% level. Pieters-Wheller Early Blood Turnip beets had significantly less PCA than Rohnert-Early Wonder Tall Top, Rohnert-

Detroit Dark Red 1-E4-11, and Robson-Seneca Detroit 1675 L varieties.

Flavor evaluation of the beets was not conducted as the total PCA, and range in PCA content among the varieties was considered too small to be responsible for any flavor defect, or preference. Shallenberger *et al.* (1959) have shown that differences in concentration of about 50 mg of PCA per 100 g of purée may cause a significant flavor difference when the PCA concentration approaches or exceeds 200 mg of PCA per 100 g of purée.

Shallenberger and Sayre (1960) have shown that beets grown without additional nitrogen fertilizer had roughly one-half as much PCA when processed as beets grown with 112 lb of actual nitrogen added per acre; the soil type was not indicated. Mahdi (1959) reported higher PCA levels in processed beets grown on sandy soils than in the same varieties at the same maturity level grown on muck soils; the fertilization practices were not indicated. The beets used in this study were grown on muck soils without nitrogen fertilization. The results compare favorably with those of Mahdi (1959). He found the average PCA content of the beets grown on three muck plots to be 106.3, compared to 104.9 mg PCA per 100 g drained weight of processed beets obtained in this study. Mahdi (1959) found the average PCA content of beets grown on five sandy soil plots to be 160.6 mg per 100 g drained weight. The work of Mahdi *et al.* (1959, 1961) and Shallenberger *et al.* (1959) and Shallenberger and Sayre (1960) shows that soil type and rate of nitrogen fertilization affect the ultimate PCA content of the processed beets. With these variables in mind, PCA levels in the varieties of beets used in this study might have been different had they been grown on sandy soil and/or with nitrogen fertilizer at levels commonly used.

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The Absorption of Sulfur Dioxide by Fruit Tissue

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(Manuscript received November 11, 1963)

SUMMARY

The influence of many individual factors on the uptake of sulfur dioxide by apricot, peach, and pear tissue before drying was tested in equipment designed to permit accurate control over sulfuring conditions. Exploratory trials showed that estimates of sulfur dioxide uptake were unreliable unless standardized sampling methods were used. Absorption rate was affected appreciably by type of fruit, surface area as influenced by size, and the use of irrigation, and less so by variety and maturity. Sulfur dioxide uptake through the skin was slow. Combinations of gas concentration and exposure time were the most important processing variables affecting absorption. Variation in air speed and temperature had little influence upon absorption. Holding cut fruits for up to 10 hr before sulfuring did not cause a decrease in gas uptake. Excess water on the fruit surface from spraying tended to decrease absorption, while steam-blanching retarded it appreciably. Any disorganization of tissue, whether from increasing maturation, storage, or processing, retarded sulfur dioxide uptake, probably through sealing of penetration pathways. The ready desorption of sulfur dioxide from tissue removed from the sulfuring environment indicated low chemical fixation at that stage.

INTRODUCTION

Exposure of apricots, peaches, and pears to sulfur dioxide before they are dried reduces deteriorative changes which otherwise occur in the fruit during and after drying. These changes, mainly in color and flavor, arise from the "browning" reaction.

Most countries prescribe maximum limits for sulfur dioxide in dried tree fruits. In Australia, a maximum of 3000 ppm is permitted. This level ensures good quality in the freshly dried product and maintains acceptability for up to a year's storage at ambient temperatures. In commercial practice, gaseous sulfur dioxide produced by burning sulfur passes into an enclosure where prepared fruit is spread on stacked trays. Equipment and procedures vary widely, resulting in sulfur dioxide levels from below 1000 ppm to over 6000 ppm. Darkening occurs rapidly in dried fruit if the level is too low, while efforts to reduce excessive concentration, mainly by leaching, result in damage to the dried material.

The problem of control of sulfuring has been investigated by numerous workers studying the drying of tree fruits, but investigations in California, Australia, South Africa, and Argentina have dealt mainly with field practice and equipment. Since the review by Long *et al.* (1940), relatively few papers have appeared on sulfuring and, although the literature reveals a wide divergence of views on the relative importance of the many variables associated with the process, no critical assessment of these factors appears to have been made.

This paper reports more precise data than have hitherto been available on factors affecting the absorption of sulfur dioxide by fruit tissue. Although studies were restricted to the more accurately controlled conditions of the laboratory, improved control of commercial sulfuring may result from the application of such information.

MATERIALS AND METHODS

Equipment. Fig. 1 illustrates equipment for sulfuring fruit under controlled conditions. It consists of ductwork in which a mixture of air and sulfur dioxide circulate. Test fruit was exposed in the upper duct, where drop-down doors with quick-acting catches allowed it to be introduced and removed rapidly. Construction was of waterproof plywood glued to a wooden frame, with some parts of stainless steel. All surfaces were coated with sulfur-resisting lacquer or epoxy

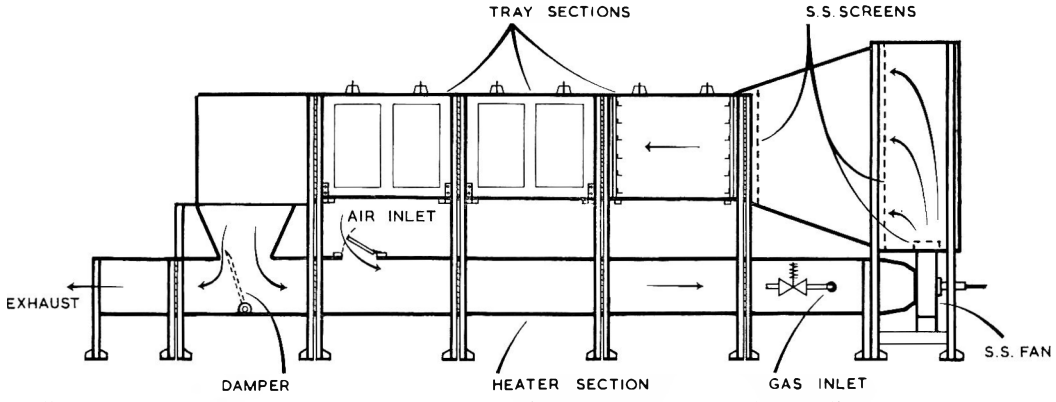

Fig. 1. Line diagram of equipment used to sulfur fruit under controlled conditions.

resin to protect against corrosion by moist sulfur dioxide. Neoprene jointing material reduced leakage between sections and around doors, but a fume hood was installed above the tunnel to remove escaping traces of gas, since this is objectionable even in low concentration.

A centrifugal fan ensured uniform mixing of sulfur dioxide in the air stream. Air speeds between 60 and 240 lin. ft/min were obtained by varying pulley size on the fan drive. Variation in air speed in the cross section of the upper duct, as measured by a hot-wire anemometer, was less than 10%. This uniformity was largely due to the resistance provided by three 30-mesh, 31-gauge, stainless-steel screens situated in the plenum between the fan and the tray sections.

A stainless-steel heater section in the lower duct housed eighteen 300-watt aluminum-clad heaters, protected against corrosion by a coating of thermoset resin. Temperature could be controlled up to 80°F above ambient, within limits of ±2°F.

Control of gas concentration. The concentration of sulfur dioxide in air within the tunnel could be controlled between 0 and 10%, within limits of ±0.1%, by a thermal-conductivity recorder-controller. Gas from a storage cylinder, reduced in pressure to ¾ in. Hg, was admitted to the tunnel just in front of the fan, through a pneumatically operated control valve. The controller was checked by a Haldane gas-analysis apparatus using potassium hydroxide as absorbent; or by an interferometer that measured the refractive index of the sulfur dioxide–air mixture. Recirculation was about 95%, and expelled gas was absorbed in a water-mist scrubber before passing to waste.

Fruit preparation. Test fruit was drawn from many growing areas, although the majority was from coastal orchards near the laboratory. Apricots and peaches were usually picked within two days of being fully ripe and were held at 20°C for further ripening. Pears were picked at commercial maturity, held at 1.1°C until required, and then ripened at 20°C. Ripe fruit was held at 1.1°C for periods which were as short as possible, seldom exceeding 7 days.

In Australia, most freestone peaches and pears are dried without peeling, but skin removal greatly improves the quality of the dried fruit. For this work, freestone peaches were steam-peeled, halved, and pitted, and cling peaches were halved, spoon-pitted, and lye peeled. Pears were steam-peeled, halved, and cored. Apricots were halved and pitted according to commercial practice.

Fruit on slatted wooden trays was placed in the tunnel immediately after preparation. The trays were supported on stainless-steel frames.

Penetration. On occasions, the penetration of sulfur dioxide into fruit tissue was studied by embedding thin-walled, open-ended glass cylinders (1-in. int diam by 1-in. long) into the flesh of fresh fruit, and trimming away excess tissue. This left a plug of fruit firmly fixed at one end of each tube, with a flat surface flush with the end. The other end was sealed to a glass slide so that, on exposure to sulfur dioxide, absorption occurred only through the prepared surface. After sulfuring, the tubes were removed from the slides and the plugs of fruit extruded by means of calibrated plungers, enabling discs 1.6 mm thick to be sliced successively from each plug. The sulfur dioxide contents of these discs showed the pattern of penetration of gas into the fruit.

Analytical methods. Samples consisting of at least 20 halved apricots, or quartered peaches or pears, were selected at random immediately after completion of a test run.

Results, to be discussed later, emphasized the need for carefully standardized sampling procedures. Consequently, fruit was always sampled from trays into a blender bowl within 60 sec of its removal from the test atmosphere. Blending commenced within 10 sec of the completion of

sampling, and analysis for sulfur dioxide was begun within 5 min.

Total sulfur dioxide was determined by the Monier-Williams method, as modified by Shipton (1954). Duplicate samples of about 15 g of blended tissue were weighed into tared, wide-necked weighing bottles having glass side-hooks, and these were lowered into the distillation flasks.

Absorption values in this paper refer to total sulfur dioxide. Methods for the estimation of "free" or uncombined sulfur dioxide in fruit slurries were not satisfactory, owing to the presence of other reducing substances.

Solids content was determined by weighing approximately 15 g of fruit slurry into a tared aluminum dish and drying in an air oven at 70±1°C for 24 hr, followed by 16 hr in a vacuum oven at the same temperature.

RESULTS AND DISCUSSION

Tests on the sulfuring equipment showed variations of up to 5% in absorption between samples of the same fruit in different runs. Samples from different trays within a run varied up to 3%, while similar differences occurred between samples taken from the same tray. Such differences, due probably to many minor variables and their interactions, indicate the degree of accuracy of the observations.

Early in the investigations, direct comparisons showed dried fruits sulfured with gas from a cylinder to be indistinguishable from samples treated by burning sulfur, thus confirming observations by Anderssen (1929); Chace *et al.* (1933); and Long *et al.* (1940).

Loss of sulfur dioxide. Fig. 2 shows the rate of loss of sulfur dioxide from peeled, halved pears standing in still air at about 70°F after removal from the sulfuring environment. This indicates that serious errors can arise from even short delays in sampling after the completion of a test.

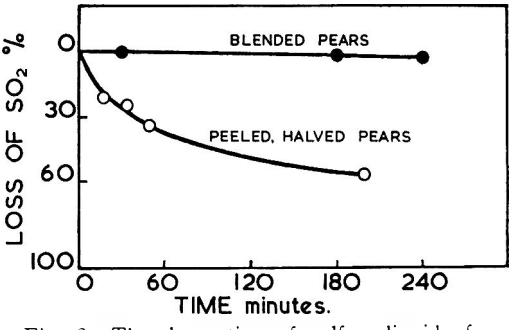

Fig. 2. The desorption of sulfur dioxide from peeled, halved pears and from blended, peeled, halved pears standing in still air at 70°F after sulfuring.

Pears from the same sulfuring run were blended for 1 min, and the slurry was allowed to stand under identical conditions. As Fig. 2 shows, the slurry lost sulfur dioxide much more slowly, losing less than 1% during 30 min, and only 4% in 240 min.

Losses from other kinds of fruit, unblended and blended, followed patterns similar to those for pears.

Frequently it would have been convenient to hold sulfured fruit samples overnight before determining their sulfur dioxide contents. However, holding samples for 16 hr at 0°C in wax-sealed, glass-stoppered jars resulted in sulfur dioxide contents 5–10% lower than those determined immediately after sampling. Such losses may be due to desorption, or to the formation of compounds in the fruit tissue from which sulfur dioxide was not released during the Monier-Williams distillation. Long *et al.* (1940) held samples of sulfured fruit in sealed jars for 1–4 days at ambient temperatures before determining their sulfur dioxide contents, and stated that losses during that interval were insignificant. Our results do not confirm this.

Depth of penetration. The penetration of sulfur dioxide into fruit tissue during sulfuring was studied to try to explain the rapid loss of the preservative from freshly sulfured fruit. Discs were cut from plugs of sulfured fruit and their sulfur dioxide content determined as described in Materials and Methods. Fig. 3 shows results with Golden Queen clingstone peaches exposed to 1% sulfur dioxide for 1 hr.

Since the mean sulfur dioxide concentration in the top 1.6 mm was almost 3600 ppm, the level at the surface must have been considerably higher. It follows that a condition favoring rapid loss of gas to the atmosphere exists immediately after sulfuring. Similar steep gradients also occurred with apricots and peaches.

Plasmolysis occurring during sulfuring of these cylinders of fruit, first at the surface and gradually extending into the lower layers, produced a translucent region which

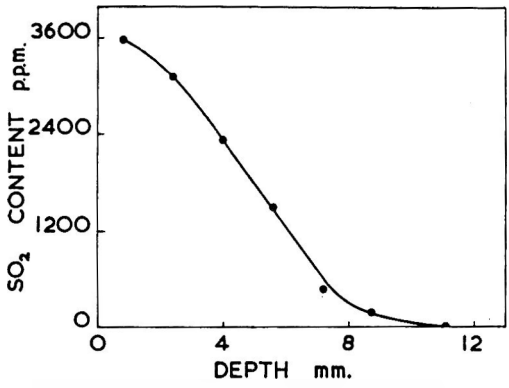

Fig. 3. A typical concentration gradient showing sulfur dioxide at various depths in clingstone peach tissue following its exposure for 1 hour at 1% gas level.

lagged well behind the gas penetration. The extent of this translucent region has often been suggested as an index of the adequacy of sulfuring. However, Chace *et al.* (1933) compared the extent of such zones in freshly sulfured apricots with sulfur dioxide levels when the fruit was dry and found the method unreliable. Our observations support their findings.

Skin penetration by sulfur dioxide. The extent to which the skin acts as a barrier was investigated by exposing whole fruits and halved, unpeeled fruits of the same size under a given set of sulfuring conditions. Sulfur dioxide absorbed by whole fruit must be taken up entirely through the skin, while the amount absorbed by halved fruits is the sum of that taken up through the skin and the cut surfaces, respectively. Since the skin area was twice that of the cut surfaces (ignoring the effect of pit cavity) the amounts absorbed through each type of surface were calculable.

The results showed that sulfur dioxide was absorbed 7–8 times faster through cut freestone peach and pear tissue surfaces than through the skins of these fruits. With apricots, cut flesh absorbed only 2.5–3 times the amount taken up through the skin, possibly because apricot skin is much thinner than that of peaches and pears.

When peeled and halved peaches were tested, the peeled flesh (from which skin was removed after being loosened by 15–20-sec exposure to steam) absorbed sulfur dioxide at a rate about 5% lower than the cut flesh. This result shows that disorganization of tissue results in decreased absorption.

Fig. 4 shows the practical effect of peeling on sulfur dioxide uptake for a range of sizes of halved freestone peaches.

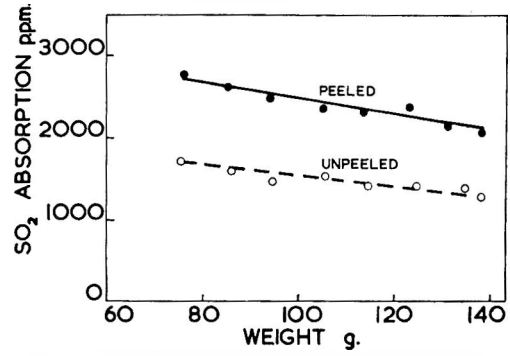

Fig. 4. The difference in sulfur dioxide absorption between peeled and unpeeled freestone peaches of various sizes after exposure for 1 hour at 1% gas concentration.

Storage prior to sulfuring. The decision to store ripened fruit for a maximum of 7 days at 1.1°C, as described earlier, followed from experiments on the influence of prestorage on absorption. Trevatt apricots were ripened and size-graded, and fruits of three individual sizes were held at 1.1°C for up to 29 days. At intervals (Table 1), samples of each size were removed from storage and sulfured for 2 hr at 2% gas concentration. Small increases—up to 2%—occurred in solids content, owing to water loss during storage.

Table 1. The influence of storage time at 1.1°C on absorption of sulfur dioxide by apricots of 3 size ranges (2 hr at 2% SO_2).

Storage time (days)	Sulfur dioxide absorption (ppm)		
	Size: 25–30 g	40–45 g	55–60 g
0	3080	2640	2410
6	3020	2600	2490
16	3000	2680	2490
29	2410	2080	1860

Results in the table are expressed on the basis of the initial level of 15% solids. They show that absorption was not markedly influenced by storage up to 16 days, but that at 29 days it was appreciably lower. By this time the fruit had softened consider-

ably, and decreased uptake might have been caused by blocking of intercellular spaces as a result of tissue breakdown. A similar decrease in absorption was noted in freestone peaches after holding them at 1.1°C for 21 days. These results showed that the storage of test material at 1.1°C for 7 days before sulfuring was unlikely to introduce significant error into the trials.

The storage of William pears for 1 month at 1.1°C before ripening did not influence uptake compared with similar fruit which was ripened without holding.

Holding prepared fruit before sulfuring. Apricots were cut and held at 95–100°F for periods up to 10 hr before sulfuring for 6 hr at 2% gas concentration. Table 2

Table 2. The influence on absorption of holding cut apricots for various times at 95–100°F before sulfuring.

Holding time (hr)	SO_2 content (ppm wet basis)	Solids content (%)	SO_2 content (ppm water-free basis)
0	6320	17.7	35,710
2	6270	19.4	32,320
4	6660	20.6	32,330
6	6690	19.8	33,790
8	7020	19.9	35,280
10	6900	20.4	33,840

shows that absorption was not decreased by such holding times. Small evaporative losses occurred during holding, as shown by a slight rise in solids content. For comparative purposes, the uptakes are also expressed on a water-free basis. Jewell (1927a) stated that absorption of sulfur dioxide was influenced by the amount of water on the fruit surface, and accordingly suggested a maximum holding time of 2 hr. Beekhuis (1936) recommended that fruit should not be held for more than 1.5 hr between cutting and sulfuring, because of the possibility of drying of the surface. Although the time between cutting and sulfuring fruit should be kept to a minimum, the results reported here demonstrate that there is no reason why cut fruit could not be held for up to 10 hr if necessary.

Christie and Barnard (1925), Jewell (1927a), and Anderssen (1929) advocated sprinkling cut fruit surfaces with water to increase gas uptake, but many other investigators found no such effect. In this investigation, spraying with water reduced absorption slightly, suggesting that the presence of water on the surface interfered with sulfur dioxide penetration.

Influence of fruit characteristics. *Size.* For a given load of fruit, the total surface or absorbing area is larger, the smaller the fruit. This is shown in Fig. 5 for peeled

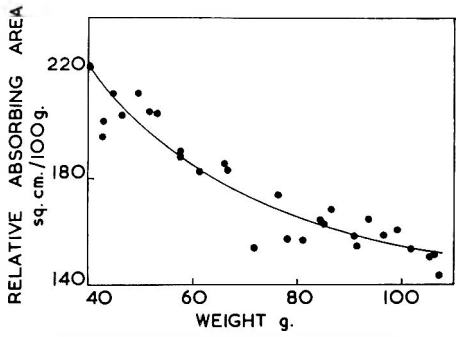

Fig. 5. The influence of fruit size on the relative surface area of peeled, halved Halehaven peaches.

Halehaven freestone peaches. The surface areas of peaches of different weights were estimated by halving, pitting, and making tracings for each fruit of: a) the cut surfaces, neglecting the pit cavity, and b) the skin, which was removed carefully after being loosened by steam for 10–15 sec. Areas of the tracings were determined by a planimeter. Actual values (Fig. 5) agreed closely with those obtained by assuming that the peaches were perfect spheres and, when halved, had a total absorptive area of $2\pi r^2 + 4\pi r^2$ where r was the mean radius.

A number of such peeled, halved fruits were then exposed to 1% sulfur dioxide for 1 hr, and the sulfur dioxide absorbed was determined. Fig. 6 shows the sulfur dioxide uptake per unit weight as a function of increasing size.

These results demonstrate that size grading of fruit is essential for reliable comparative tests. In attempting to demonstrate the influence of size on sulfur dioxide uptake by apricots, Long *et al.* (1940) separated fruits of uniform maturity into 3 size grades: over 2 in., 1.5–2 in., and under 1.5 in. in diameter. Although their results showed a slight increase in absorption with

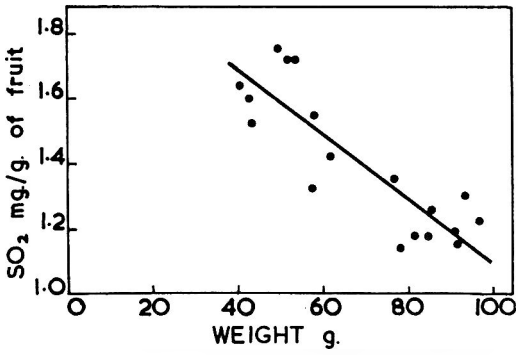

Fig. 6. The decrease in absorption of sulfur dioxide per unit weight with increasing size for peeled, halved, freestone peaches.

decrease in size, differences were too small to be conclusive. In the present work, grading was by weight: grade differences were 5 g for small fruit and 10 g for large fruit.

Maturity. Three maturity grades of apricots, peaches, and pears were tested. The first grade was "ripe," indicating full color; the second was "half-colored," needing 3–4 days at 20°C to ripen; the third was "immature," mostly green fruit needing 8–10 days at 20°C to ripen.

Table 3 shows the results of two typical tests in which clingstone peaches and pears of each maturity were sulfured for 1 hr at 1% sulfur dioxide. Absorption decreased slightly but consistently as maturity increased. These experiments demonstrated that variations in absorption due to maturity are unimportant in comparative trials when ripe fruit are used. After allowing for size and maturity of the fruit, residual variations did not exceed 5%.

Uncertainty about the influence of maturity on sulfur dioxide uptake existed until Long *et al.*, in 1940, obtained results similar to those demonstrated in this paper. The uncertainty arose from observations that "ripe" fruit, when dried, contained more sulfur dioxide than similarly processed immature fruit. Some workers failed to realize the significance of differences in desorption which occur during drying.

Type of fruit. The uptake of sulfur dioxide by different types of fruit was studied, using halved fruits (when identical sizes occurred) or plugs of tissue, as previously described. On a few occasions, samples of simultaneously maturing fruit were exposed in the same sulfuring run, but most comparisons were made in separate, though practically identical, runs.

The most rapid absorption of sulfur dioxide was by apricot tissue; cling peaches took up the gas at a slightly faster rate than freestone peaches; while pears had by far the slowest absorption. This order of uptake is in general agreement with the findings of Long *et al.* (1940), as indicated by their "absorption factor."

Irrigation. Apricots and peaches grown under irrigation absorbed sulfur dioxide at a faster rate than similar unirrigated material. The difference was more pronounced with apricots than peaches, as shown in Table 4. The influence of irrigation on gas uptake by pears was negligible.

The cut surfaces of irrigated apricots, in particular, and to a less extent those of irrigated peaches, were visibly more porous

Table 4. Differences in sulfur dioxide uptake between irrigated and unirrigated fruit (2% SO_2).

Type	Sulfuring time (hr)	SO_2 absorption (ppm)	
		Irrigated	Unirrigated
Apricots	4	4790	3520
(size 45–50 g)	6	5790	4120
	8	6660	4500
Freestone			
peaches	2	2350	2100
(size 200–210 g)	4	3000	2560
	8	3510	2910

Table 3. The influence of maturity on the sulfur dioxide absorption by peaches and pears (1 hr at 1% SO_2).

Type of fruit	wt (g)	SO_2 absorption (ppm)		
		Ripe	Half-colored	Immature
Peeled, halved cling peaches (var Golden Queen)	110	1600	1680	1700
Peeled, halved pears (var Winter Cole)	140	990	1020	1080

than those from unirrigated fruits, and it is suggested that penetration was more rapid in the former.

Variety. Variety did not have an important influence on the absorption of sulfur dioxide. Comparisons showed that, for several kinds of fruit, varietal samples of similar size and maturity grown under similar conditions absorbed sulfur dioxide at almost identical rates. These observations were made on varieties grown most widely in Australia for drying, and included Trevatt, Moorpark, and Royal apricots; Elberta, Blackburn Elberta, J. H. Hale, and Halehaven freestone peaches; and Williams Bon Chrétien and Winter Cole pears.

Most early investigators agreed that variety affected sulfur dioxide uptake, but there is little published evidence to support this view. Long *et al.* (1940) had results suggesting that variety affects absorption, but other variables, such as growing conditions and sulfuring procedures, could have had an influence.

Influence of processing variables on gas uptake. *Air speed.* A series of tests in which air speed over the fruit during sulfuring was varied from 60 lin. ft/min (just-perceptible air movement) up to 240 lin. ft/min (gentle breeze conditions) showed that gas uptake was not influenced by such changes. Air speed was usually standardized at 100 lin. ft/min, which caused only slight evaporation from the fruit during a run, as shown by moisture determinations. This was probably due to the high recirculation rate of the air-gas mixture, only a small bleed to waste being necessary to maintain steady control.

Temperature. Although Chace *et al.* (1930) found that, over the range 80–140°F, temperature had little influence on the absorption of sulfur dioxide by fruit tissue, several workers have reported otherwise (Christie and Barnard, 1925; Quinn, 1926; Jewell, 1927b; Nichols and Christie, 1930; Long *et al.*, 1940). In the present work it was found that temperature had no consistent effect during sulfuring. This is illustrated in Table 5, which shows results for pears between 80 and 130°F. Results were similar with apricots and peaches.

Table 5. The influence of temperature on absorption of sulfur dioxide by pear tissue (1 hr at 1% SO_2).

Temperature of sulfuring (°F)	SO_2 absorption (ppm)
80[a]	1080
90	1000
100	1030
110	1020
120	1070
130	1010
80[a]	1020

[a] 80°F tested on first and seventh day of trial.

Concentration. Sulfur dioxide concentration in the atmosphere surrounding the fruit was one of the most important factors influencing the uptake of gas. Preliminary tests showed that the important practical levels of gas concentration were 1–3%, i.e. near the lower end of the range of our control equipment. Long *et al.* (1940) found that gas concentrations seldom exceeded 3% in commercial practice.

Fig. 7 shows that, for peaches and apricots, proportionally less sulfur dioxide was absorbed at higher gas concentrations. The sulfur dioxide uptake by peeled freestone peaches over a range of sizes is shown in Fig. 8 for two gas concentrations.

Time of exposure. Absorption increases with time are illustrated in Fig. 9 for unpeeled freestone peaches. The rate was greatest immediately after the start of the run, and gradually decreased. These peaches absorbed four times as much sulfur dioxide in the first 12 hr as in the following 16 hr. All other fruits reacted in a similar manner.

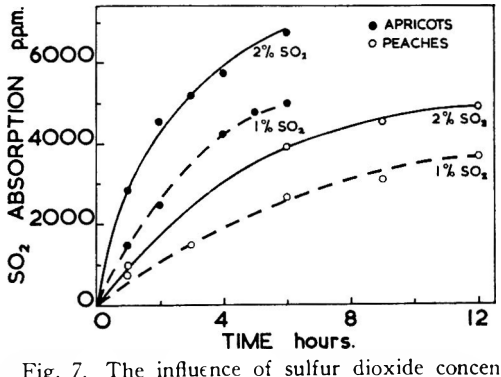

Fig. 7. The influence of sulfur dioxide concentration on the rate of gas uptake by apricots and peaches.

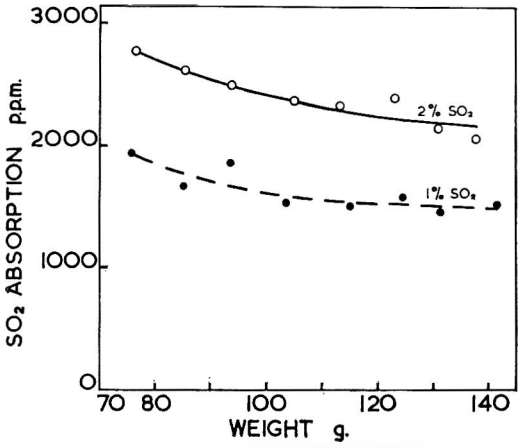

Fig. 8. The amounts of sulfur dioxide absorbed by peeled freestone peaches of various sizes after 1 hour exposure at 1 and 2% gas concentration.

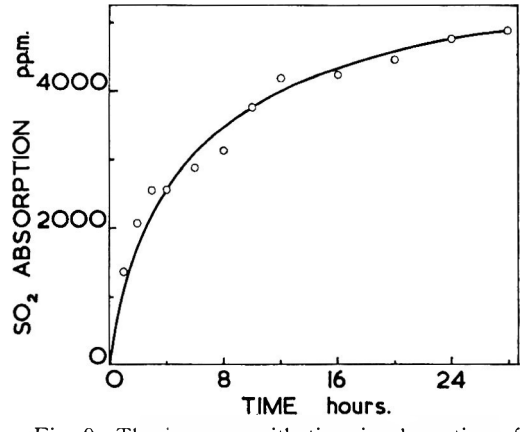

Fig. 9. The increase with time in absorption of sulfur dioxide by unpeeled, halved, freestone peaches at a gas concentration of 1%.

Sulfuring time being the easiest factor to control, many investigators have made specific recommendations for each type of fruit. These recommendations differ widely, e.g. Hiltner (1928, unpublished data) concluded that apricots and peaches could be sulfured in 3 hr, while Cruess (1938) suggested up to 72 hr for pears. Many proposals combine time of exposure with the weight of sulfur to be burnt per unit weight of fruit, but, owing to variations in burning rate, the amount of sulfur consumed bears little relationship to the length of burning period (Lyon, 1930). The "gas environment value" of Long *et al.* (1940), which is the sulfuring time in minutes multiplied by the mean gas concentration during the run, is of doubtful utility, owing to the difficulty of measuring the mean gas concentration.

Blanching. Blanching in live steam before sulfuring has been recommended by a number of American investigators for producing fruit that is translucent when dried. Nichols and Christie (1930) and Chace *et al.* (1933) reported that this treatment did not affect sulfur dioxide absorption. We, on the contrary, have found that blanching causes a marked reduction in uptake. For example, unblanched peeled and halved freestone peaches took up 1910 ppm, while identical fruit blanched for 5 min and cooled before sulfuring absorbed only 1610 ppm. Since the water content of the fruit was not altered significantly by blanching, it is suggested that the considerable disorganization caused by this treatment reduces the pathways by which sulfur dioxide may enter the tissue.

CONCLUSION

The foregoing results show that fruit characteristics that influence sulfur dioxide absorption are size, type, and whether the fruit has been grown under irrigation. The nature of the exposed fruit is also of importance, and, because skin acts as a barrier to sulfur dioxide penetration, peeling before sulfuring should be advantageous except where, as with apricots, peeling causes disintegration.

It is clear that factors tending to cause disorganization of the surface tissue retard the rate of sulfur dioxide absorption, and perhaps even the total uptake. Thus, reduced uptake was obtained with fruit sprayed with water or blanched with steam before sulfuring, and over-mature and long-stored fruit showing tissue breakdown absorbed less sulfur dioxide than firm, fresh fruit. These observations suggest that gaseous diffusion mechanisms are more important than solution in the liquid phase, or chemical combination with the tissue, although, no doubt, these also occur during sulfuring. Disorganization of tissue by plasmolysis presumably leads to the blocking or breakdown of intercellular pathways for the gas, thus adversely affecting its diffusion into the tissue.

Process variables having the greatest influence on sulfur dioxide absorption were

found to be gas concentration and the time the fruit was exposed to the sulfuring atmosphere. Fig. 9 shows that, after 28 hr, gas uptake by peaches had almost leveled out at about 5000 ppm when the gaseous environment was 1% in sulfur dioxide, whereas, with apricots, Long *et al.* (1940) noted a maximum absorption of about 4500 ppm during commercial sulfuring. It is probable, therefore, that the very long sulfuring times sometimes advocated are not necessary.

Provided that the concentration of sulfur dioxide in the surrounding atmosphere can be maintained reasonably constant during sulfuring, the time of exposure of the fruit to the gas offers the most convenient means of regulating the uptake of sulfur dioxide by the fruit. However, the control of sulfur dioxide concentration in present commercial equipment is almost impossible, because the sulfur burning rate is altered by changes in wind speed and direction, resulting in wide fluctuations in gas concentration within the enclosure. If the cost of cylinder sulfur dioxide cannot be borne, the development of sulfur-burning equipment capable of supplying sulfur dioxide at a steady rate seems warranted.

Regulated absorption of sulfur dioxide is only the first requirement for producing dried tree fruits having a particular sulfur dioxide content, because desorption commences immediately after fruit is taken from the sulfuring atmosphere. Further studies on this aspect of the problem would doubtless be of value.

ACKNOWLEDGMENT

Grateful acknowledgment is made to Mr. A. Howard, who initiated the search for control equipment for sulfur dioxide in air, and to Mr. J. Wallace, for technical assistance and preparation of graphs.

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RESEARCH NOTE

Further Investigations on the Properties of the 2,4-Dinitrophenylhydrazine Derivatives of Diacetyl, α-Acetolactic Acid, and Acetoin[a]

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An earlier communication (Lindsay *et al.*, 1962) reported some of the properties of the 2,4-dinitrophenylhydrazine (DNP) derivatives of diacetyl, α-acetolactic acid, and acetoin. Subsequent work has revealed that the compound originally reported as the DNP-hydrazone of acetoin is 2,4-dinitroaniline. This communication corrects the earlier data and relates some additional properties of the derivatives that enable their identification.

The revised and corrected properties of authentic derivatives are given in Table 1. The properties for diacetyl DNP-hydrazone and diacetyl bis-(DNP-hydrazone) are as reported previously. It should be noted, however, that the data presented for 2,4-dinitroaniline were previously reported as that for acetoin DNP-hydrazone. The properties of the acetoin DNP-hydrazone are in agreement with those reported by other workers (Patton *et al.*, 1958; Day *et al.*, 1960), and contrary to our earlier communication.

Infrared spectra are useful for distinguishing the derivatives provided special care is taken in purification and drying of materials. After chromatography of the DNP-hydrazones as described previously, the acetoin DNP-hydrazone was further purified by a hexane-nitromethane partition column (Day *et al.*, 1960) and recrystallized from hexane. Diacetyl bis-(DNP-hydrazone) was purified by chromatography on a benzene-ethanol amine partition column (Schwartz, 1962). To circumvent the interfering OH stretch due to water, the DNP-hydrazones were dried at 60°C at pressure of 30 mm Hg for 24 hr prior to analysis. The KBr used in making pellets was ground for 1 min in a stainless-steel capsule and then dried 24 hr at 360°C. All samples were handled rapidly immediately prior to analysis to prevent adsorption of excessive moisture.

The distinguishing characteristics of the IR spectrum for the diacetyl bis-(DNP-hydrazone) (Fig. 1a) are the absence of significant OH or NH stretch and C=O stretch. Diacetyl DNP-hydrazone (Fig. 1b) shows the absence of the significant OH or NH stretch, but shows a relatively intense C=O stretch band near 1685 cm^{-1}. Acetoin DNP-hydrazone (Fig. 1c) shows an intense band near 3380 cm^{-1}, but does not show a C=O stretch band. The band near 3380 cm^{-1} for acetoin DNP-hydrazone is a rather broad band, which is to be expected where hydrogen-bonding through a hydroxyl group

[a] Supported by funds granted by the American Dairy Association.

Table 1. Properties of 2,4-dinitrophenyl hydrazine derivatives.

Derivative	Melting point (°C)	λmax Chloroform	λmax Alcoholic KOH	Color in Alcoholic KOH
Diacetyl bis-(DNP-hydrazone)	318d	430,394	540	Purple
Diacetyl DNP-hydrazone	175	355	500	Reddish pink
Acetoin DNP-hydrazone	114	357	500	Red
2,4-Dinitroaniline[a]	178–9	327,259	510,382	Bright pink

[a] Soluble in water.

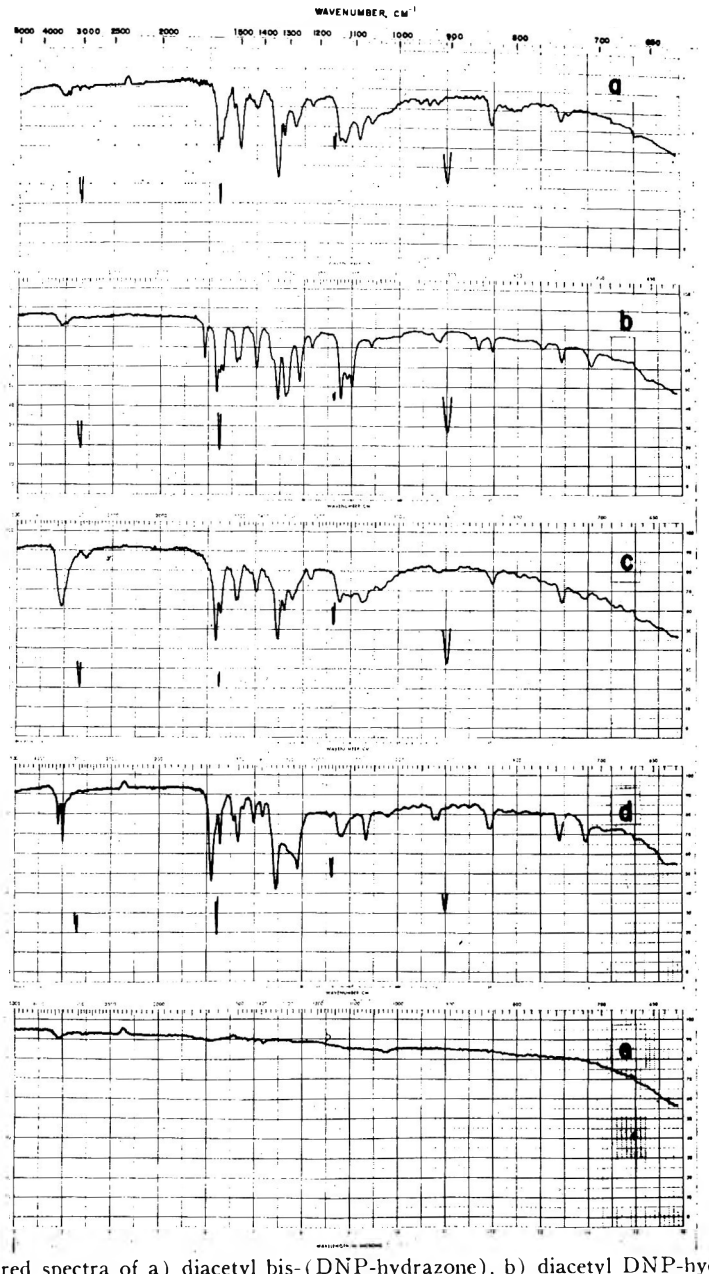

Fig. 1. Infrared spectra of a) diacetyl bis-(DNP-hydrazone), b) diacetyl DNP-hydrazone, c) acetoin DNP-hydrazone, d) 2,4-dinitroaniline, e) blank KBr pellet.

can be effected. The IR spectrum for 2,4-dinitroaniline (Fig. 1d) shows two sharp bands near 3333 cm^{-1} and 3460 cm^{-1}. The two bands are characteristic of the NH stretch resulting from the two NH bonds in the free amine group.

The origin of 2,4-dinitroaniline in carbonyl reaction mixtures is probably through a series of reactions analogous to those for phenylhydrazine reacting with an α-hydroxy ketone (Holleman and Richter, 1953):

$$R\cdot CH(OH)\cdot CO\cdot R' + C_6H_5\cdot NH\cdot NH_2 \xrightarrow[\text{acetic acid}]{\text{dilute}} R\cdot CH(OH)\cdot C(:N\cdot NH\cdot C_6H_5)\cdot R' + H_2O \quad [I]$$

$$R\cdot CH(OH)\cdot C(:N\cdot NH\cdot C_6H_5)\cdot R' + C_6H_5\cdot NH\cdot NH_2 \longrightarrow R\cdot CO\cdot C(:N\cdot NH\cdot C_6H_5)\cdot R' + NH_3 + C_6H_5\cdot NH_2 \quad [II]$$

$$R\cdot CO\cdot C\cdot(:N\cdot NH\cdot C_6H_5)\cdot R' + C_6H_5\cdot NH\cdot NH_2 \longrightarrow R\cdot C(:N\cdot NH\cdot C_6H_5)\cdot C(:N\cdot NH\cdot C_6H_5)\cdot R' + H_2O \quad [III]$$

With DNP-hydrazine and acetoin, reaction I would yield acetoin DNP-hydrazone; reaction II would yield diacetyl DNP-hydrazone and 2,4-dinitroaniline; reaction III would yield diacetyl bis-(DNP-hydrazone).

The rigorous extraction of the reaction mixtures with ethylene chloride to obtain the diacetyl bis-(DNP-hydrazone) undoubtedly recovered considerable quantities of 2,4-dinitroaniline hydrochloride in the samples to be analyzed by column chromatography. Since the magnesium oxide-celite adsorption columns are quite basic, the hydrochloride salt could be readily converted to the free amine, thus accounting for its slight solubility in water and relatively short elution time from the column. Since 2,4-dinitroaniline has appeared as a component in derivatives from several autoxidizing fat samples in this laboratory, it is well that other workers in this area be aware of its potential presence in unknown DNP-hydrazone mixtures.

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Tocopherol Distribution in Milk Fractions and Its Relation to Antioxidant Activity[a]

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SUMMARY

Studies were made of the distribution of tocopherol between the lipid in the fat globule membrane and inside the milk fat globule, and the relation of that distribution to the loss of tocopherol during lipid oxidation. Two fractionation procedures were used, one based on churning of cream, the other on washing cream by repeated dilution with sucrose-saline solution and separation, followed by treatment with sodium deoxycholate. The tocopherol concentration was at least three times higher in the lipid of the fat globule membrane than inside the fat globule. During lipid oxidation, the tocopherol associated with the fat globule membrane was lost more rapidly. The results demonstrated that, in determining the oxidative stability of milk, the membrane tocopherol is more important than that inside the fat globule.

INTRODUCTION

Oxidized flavor in milk is attributed to oxidation of phospholipids (Thurston *et al.* 1937; Swanson and Sommer, 1940; Smith and Dunkley, 1959), which are concentrated in the fat globule membrane. The fatty acids of the phospholipids are more unsaturated, and hence more susceptible to oxidation, than are those of the triglycerides (Lea, 1957; Smith and Jack, 1959). Also, the lipids in the fat globule membrane are more closely associated with prooxidants such as copper and ascorbic acid (Smith and Dunkley, 1962).

In most studies of the oxidation of lipids or tocopherol in milk and milk products, milk fat has been obtained by churning or treatment with a detergent. These methods isolate the bulk of the triglycerides, but not the phospholipids in the fat globule membrane (Stine and Patton, 1953). The results of such studies could be misleading because changes would be undetected in the most labile fraction, the unsaturated lipids of the fat globule membrane.

Frankel *et al.* (1958) isolated lipids from dried buttermilk by solvent extraction and found a tocopherol content of 72 μg/g, which is higher than normal for milk fat. They also found a higher concentration of tocopherol in lipids isolated by solvent extraction of cream than in lipids isolated by churning. Those results indicate that tocopherol is present in higher concentration in the lipids in the fat globule membrane than in the triglycerides, which make up the bulk of the fat globule.

This report describes a study of the distribution of tocopherol between the lipid in the fat globule membrane and inside the globules, and of the loss of tocopherol during lipid oxidation.

EXPERIMENTAL METHODS

Except where specified otherwise, the milk, methods of sampling, and analytical procedures were as in previous studies (Erickson and Dunkley, 1963; Erickson *et al.*, 1963). Lipid phosphorus was by the method of Smith *et al.* (1959), protein by a semimicro-Kjeldahl method, xanthine oxidase activity by the vanillic acid method (Kuramoto, *et al.*, 1957; Smith and Dunkley, 1960), and alkaline phosphatase activity by the method of Haab and Smith (1956).

Lipid fractionation by cream churning. Fig. 1 outlines the procedure. Milk that was still warm after milking, with copper added when required, was allowed to cream 1–4 hr in a 500-ml separatory funnel immersed in an ice-water mixture.

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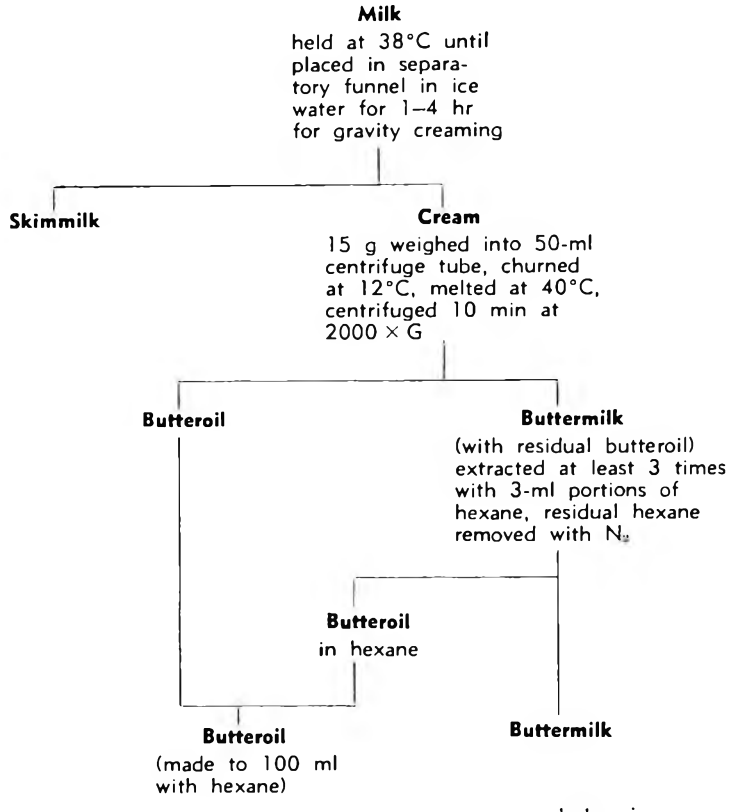

Fig. 1. Schematic outline of fractionation of milk by creaming and churning.

The skimmilk was drained off, and the cream was churned either immediately or after storage for development of oxidized flavor. About 15 g of the cream was weighed into a 50-ml centrifuge tube and churned at 12°C on a mechanical shaker. The tube was immersed 10 min in water at 40°C to melt the butter granules, and centrifuged 10 min at 2000 × G. The butteroil was melted at 40°C, and most of it was transferred by pipette to a 100-ml volumetric flask. The remaining butteroil was removed by layering 3 ml of hexane over the buttermilk, gently swirling the tube to mix the hexane with the butteroil, centrifuging briefly, and removing the hexane-butteroil layer with a capillary pipette. The hexane-layering step was repeated at least 2 more times, until the hexane layer remained colorless. The butteroil fractions were combined, and hexane was added to make the solution to 100-ml volume. The hexane remaining with the buttermilk was evaporated by a stream of nitrogen. Lipid and tocopherol were determined in the cream, butteroil, buttermilk, and, in some experiments, skimmilk.

Lipid fractionation of washed cream by deoxycholate (DOC). Reagents required were:

Sucrose-saline solution. 0.25*M* sucrose, 0.15*M* sodium chloride, buffered at pH 6.9 with 0.01*M* sodium phosphate. Solutions of the chemicals used for preparing this reagent were passed through Dowex A-1 chelating resin (Dow Chemical Co., Midland, Mich.) to remove contaminating copper.

DOC solution. 10% sodium deoxycholate (Matheson, Coleman and Bell, Cincinnati, Ohio) in 0.25*M* sucrose buffered at pH 8.5 with 0.25*M* 2-amino-2-hydroxymethyl-1,3-propanediol (Tris, Sigma Chemical Co., St. Louis, Missouri).

Fig. 2 outlines the procedure. Milk from several cows was combined and kept at 38°C until separated (De Laval Model 518). The cream was washed four times with sucrose-saline solution (i.e. the cream was diluted with four times its volume of sucrose-saline solution, then re-separated). The washed cream was fractionated immediately, or stored at 4°C for study of the tocopherol loss accompanying lipid oxidation. For the storage studies, 0.1 μg of copper and 25 μg of ascorbic acid per gram of washed cream were added to promote lipid oxidation.

The DOC treatment was essentially that of Hayashi *et al.* (1963). Briefly, 30 g of washed

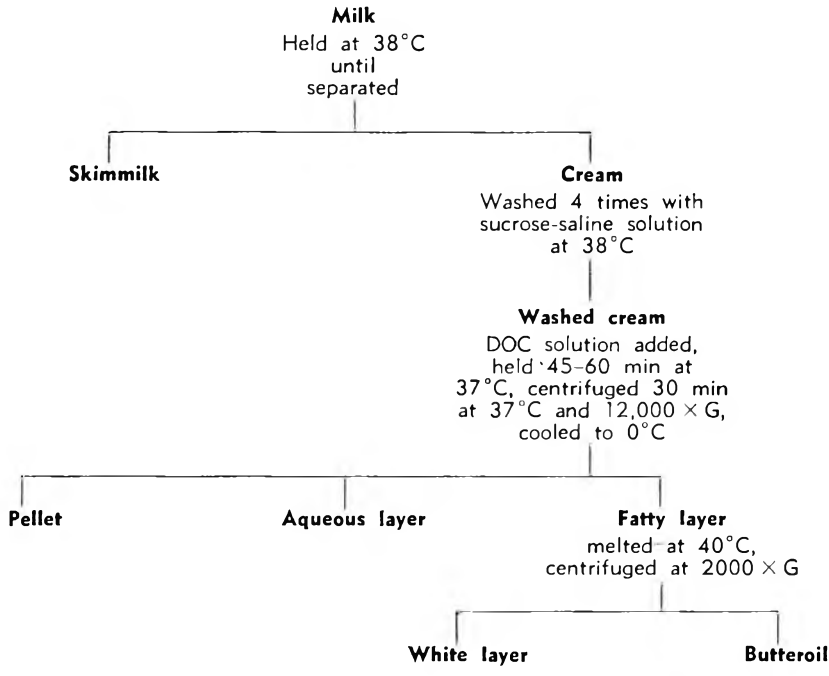

Fig. 2. Schematic outline of fractionation by washing cream and treating it with DOC.

cream was weighed into a 50-ml Lusteroid centrifuge tube, and sufficient DOC solution was added to give 1% DOC in the aqueous phase of the washed cream. The mixture was held 45–60 min at 37°C with gentle shaking, centrifuged (Servall RC2, type SS-34 rotor) 30 min at 37°C and 12,000 × G, and cooled to 0°C to solidify the fatty layer. The pellet and aqueous layer were removed. The solidified fatty layer was transferred to a 50-ml glass centrifuge tube, warmed to 40°C, and centrifuged at 2000 × G. The upper butteroil layer was removed by pipette and hexane layering, as in the fractionation by churning, leaving a white layer. Lipid and tocopherol were determined in selected fractions.

RESULTS AND DISCUSSION

Fractionation by separation and churning. Table 1 shows the distribution of lipid and tocopherol in fractions obtained from milk by gravity creaming, and by centrifugation, followed by churning of the corresponding creams. For separation by centrifugation, the procedure was as in Fig. 1 except that the milk was separated in a cream separator (De Laval Model 518) immediately after milking. Gravity creaming gave a tocopherol concentration in the lipid in the skimmilk similar to that in the whole milk and cream. In contrast, centrifugal separation gave a tocopherol concentration in the lipid that was higher in the skimmilk and separator slime than in the whole milk and cream. Churning fractionation gave a tocopherol concentration in the lipid that was higher in the buttermilk fractions than in the butteroils.

Buttermilk contains membrane lipid and also lipid from inside fat globules that are not included in the butter granules. For the gravity and centrifugal creams, respectively, the ratios of the concentration of tocopherol in the buttermilk lipid to that in the butteroil were 2.5 and 2.4. The ratios would have been higher if the buttermilk did not contain intact fat globules not included in the butter granules by churning.

The two skimmilks from the separation fractionation differed markedly in tocopherol concentration in the lipid. This result can be explained on the basis of the less complete recovery of fat from skimmilk by gravity creaming. Differences in the size distribution of fat globules in the two skimmilks, however, would also influence the tocopherol concentration in the lipid. In gravity creaming the clustering of the fat globules would result in a more or less normal distribution of the sizes of the fat globules

Table 1. Distribution of lipid and tocopherol in fractions obtained from milk by centrifugal separation and gravity creaming, and by churning of the corresponding creams.

Fractionation method	Fraction	Lipid (%)	Lipid (g)	Lipid (% of total)	Tocopherol (μg/g lipid)	Tocopherol (μg)	Tocopherol (% of total)
Separation by:							
Gravity creaming	Whole milk	4.93	29.3	.....	19.2	536	.....
	Skimmilk	0.42	1.9	6.5	17.2	33	5.9
	Cream	20.6	27.5	93.8	19.0	523	93.0
Centrifugation	Whole milk	4.93	1,780	.....	19.2	34,100	.....
	Skimmilk	0.065	20.0	1.1	33.3	670	1.7
	Cream	38.2	1,760	98.3	19.2	33,900	99.2
	Separator slime	2.80	0.6	0.03	116.0	66	0.2
Churning:							
Gravity cream	Cream	20.6	3.09	.....	19.0	58.7	.....
	Buttermilk	0.9	0.11	3.7	45.2	5.0	8.5
	Butteroil	.....	2.91	94.1	18.1	52.6	90.0
Centrifugal cream	Cream	38.2	5.73	.....	19.2	110	.....
	Buttermilk	2.1	0.20	3.5	44.0	8.8	8.0
	Butteroil	.....	5.40	94.1	18.0	97.0	88.0

in the skimmilk, but in efficient centrifugal separation the fat globules in the skimmilk would be less than 2 μ in diameter (Jenness and Patton, 1959). Furthermore, because the nonlipid constituents of the membrane are denser than the suspending skimmilk, the smallest fat globules, with the greatest surface-to-volume ratios, would be concentrated in the separator slime. These considerations explain the high tocopherol concentration in the lipid in the centrifugally separated skimmilk and slime.

Fractionation by cream washing and DOC treatment. In the preparation of fat globule membrane material, either water or a dilute buffer is commonly used to remove, by repeated dilutions and separations, the bulk of the nonfat constituents that are not associated with the fat globules (King, 1955). Some lipoproteins, however, are not stable in the presence of distilled water (Nichols *et al.*, 1961). Therefore, a comparison was made of washing with distilled water and with a sucrose-saline solution. The solution was approximately isotonic with and had a density similar to that of skimmilk.

Table 2 shows that buttermilk contained higher amounts of several constituents associated with the fat globules when it was made from cream washed with the sucrose-saline solution. These results emphasize the advantage of washing cream with sucrose-saline solution.

Table 2. Comparison of buttermilk from cream washed with distilled water and sucrose-saline solution.

	Cream washed with: Distilled water	Cream washed with: Sucrose-saline solution
TMS,[a] %	1.09	1.21
Lipid, % of TMS	41.3	39.7
Protein, mg/g TMS	379	620
Tocopherol, μg/g lipid	17.4	19.2
Copper, μg/g TMS	15.2	20.5
Xanthine oxidase, A_{640} per ml	1.64	2.82
Alkaline phosphatase, A_{600} per ml	0.118	0.133
pH	6.3	6.8

[a] Total milk solids.

As with centrifugal separation, the washing step causes some loss of fat, mainly as the smaller fat globules, which have a high tocopherol concentration in the lipid (Table 1). Loss of the smaller fat globules would result in a greater percentage loss of tocopherol than of lipid, but tocopherol could also be removed from the surface of the fat globules by washing. In a representative experiment, during separation and washing, respective losses of lipid and tocopherol were 2.5 and 19.8%.

To avoid the violent agitation of churn-

ing, which could cause destabilization of proteins and lipoproteins, the DOC treatment of Hayashi *et al.* (1963) was used to fractionate the washed cream, as in Fig. 2. Table 3 compares the distribution of lipid and tocopherol resulting from fractionation by DOC treatment and by churning. The churning fractionation was modified from that in Fig. 1 by centrifuging the mixture of melted fat and buttermilk 30 min at 37°C and 12,000 × G, as in the DOC treatment (Fig. 2). This modification made it possible to separate the buttermilk into pellet, aqueous layer, and white layer, corresponding to those fractions in Fig. 2.

The DOC treatment gave an aqueous layer-pellet fraction containing less lipid but more tocopherol than did churning. Differences were also observed in the appearance of the aqueous layers—that from the DOC treatment was clear and reddish whereas that from churning was turbid.

In interpreting the results of the DOC fractionation, it is assumed that the butteroil represents the inside of the fat globules, and the lipid in the white layer, aqueous layer, and pellet represents lipid in the membrane material. The DOC fractionation, which uses a different principle of separation, gave results generally similar to those obtained by churning. In Experiments 1 and 2, the respective tocopherol concentrations in the membrane lipid were 3.4 and 2.4 times higher than inside the fat globules. Corresponding values for the churning fractionation were 1.5 and 0.8. These values are lower than for the churning fractionation of unwashed cream.

Comparison of the tocopherol concentration in the membrane to that inside the fat globules for the washed (Table 3) and unwashed (Table 1) creams indicates that part of the loss of tocopherol from washing was from the fat globule surface, not just

Table 3. Comparison of distribution of lipid and tocopherol in fractions obtained from milk by two fractionation treatments.

Treatment	Fraction	Lipid (g)	Tocopherol (μg/g lipid)	Tocopherol (μg)
Experiment 1				
DOC	Washed cream	16.0	19.3	309
	Butteroil	15.9	17.3	274
	White layer	0.18	26.3	4.8
	Aqueous layer-pellet	0.06	153.8	9.2
	Total in fractions	16.1		288
	Recovery, % [a]	101.0		93.1
Churning	Washed cream	16.0	19.3	309
	Butteroil	15.4	16.8	259
	White layer	0.64	17.6	11.3
	Aqueous layer-pellet	0.08	87.6	7.0
	Total in fractions	16.2		277
	Recovery, % [a]	101.0		89.5
Experiment 2				
DOC	Washed cream	15.6	21.0	328
	Butteroil	15.1	20.9	316
	White layer	0.21	19.4	4.7
	Aqueous layer-pellet	0.05	156.0	8.9
	Total in fractions	15.4		329
	Recovery, % [a]	98.5		100.0
Churning	Washed cream	15.6	21.0	328
	Butteroil	14.5	20.8	303
	White layer	0.58	14.5	8.4
	Aqueous layer-pellet	0.11	28.2	3.1
	Total in fractions	15.2		314
	Recovery, % [a]	97.7		96.0

[a] Of total in cream.

from the loss of the smaller globules. For the data in Table 3, therefore, the washing would have caused loss of tocopherol from the fat globule membrane material in the DOC fractionation as well as in the churning fractionation. Hence, even the observed higher concentrations of tocopherol in the membrane lipid than inside the fat globules must be low compared with those in normal globules.

From consideration of all of the experimental data, we conclude that the concentration of tocopherol in the membrane lipid is more than three times higher than inside the fat globules.

Losses of tocopherol from fractions during lipid oxidation. Table 4 shows losses of tocopherol in milk fractions during storage under conditions conducive to lipid oxidation (See Lipid fractionation by cream churning). Tocopherol in the buttermilk fraction decreased 68% in 38 hr, whereas that associated with the butteroil did not change.

Table 5 presents similar results for washed cream treated with DOC and stored 35 hr at 4°C after adding copper and ascorbic acid (see Lipid fractionation of washed cream by DOC). Tocopherol in the white layer and aqueous layer-pellet fractions was destroyed completely, but only 30% was lost in the washed cream and butteroil.

The results in Tables 4 and 5 show that the tocopherol in the fat globule membrane is oxidized more rapidly than that inside the fat globules.

The bulk of the lipid in fat globules is inside (in the butteroil fraction) rather than in the membrane. Therefore, most of the tocopherol is inside the fat globules. However, the tocopherol concentration in the lipid is higher in the membrane than inside the globules. Furthermore, the tocopherol in the membrane is oxidized more rapidly because of the higher lability of the lipids and the proximity of prooxidants. Since the fat in milk is solid under normal storage conditions, little if any tocopherol would diffuse from inside to the membrane of the globules. Hence, tocopherol inside the globules would be ineffective in retarding oxidized flavor. As an inhibitor of oxidized flavor, therefore, the membrane tocopherol is more important than that inside the globules.

Table 4. Losses of tocopherol in milk fractions during storage of gravity cream from milk containing 0.1 μg/g of added copper.

Storage time (hr)	TBA increase[a] (A_{535})	Tocopherol in: Cream (μg)	Cream (% loss)	Butteroil (μg)	Butteroil (% loss)	Buttermilk (μg)	Buttermilk (% loss)
0	....	52.6	....	39.8	....	10.9	....
14	0.075	49.0	6.8	38.6	3.0	8.9	18.3
38	0.204	41.5	21.1	39.6	0.5	3.5	68.0

[a] Based on 9 g of cream.

Table 5. Losses of tocopherol in milk fractions during storage of washed cream 35 hr at 4°C after adding 0.1 μg/g of copper and 25 μg/g of ascorbic acid. Fractionation was by DOC treatment.

Storage time (hr)	Fraction	TBA increase[a] (A_{535})	Tocopherol (μg)	Tocopherol (% loss)
0	Washed cream	....	186	....
	Butteroil		175	....
	White layer		2.0	....
	Aqueous layer-pellet		3.3	....
35	Washed cream	0.072	128	31.2
	Butteroil		122	30.3
	White layer		0.0	100.0
	Aqueous layer-pellet		0.0	100.0

[a] Per g of washed cream.

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S. Hayashi collaborated in comparison of the churning fractionation with that by washing and DOC treatment.

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Organic Acids in Citrus Fruits. I. Varietal Differences[a]

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(Manuscript received June 11, 1963)

SUMMARY

Organic acids were determined in juices and peels of mature fruits of Washington Navel and Valencia oranges, grapefruit, lemon, tangerine, and Palestine sweet lime. Juices and peels were freeze-dried, and organic acids were determined by silicic-acid chromatography. Cation-exchange resin in the hydrogen form was used for acidification of peel prior to extraction of acids with methanol. Citric acid predominated in all juices except that of the sweet lime (in which citrate level was so low that malate exceeded it). Malate occurred in all juices in low but measurable concentrations. Oxalate predominated in the peels, but in the lime, malonate appeared in equivalent amounts. Malonate appeared in all peels in measurable amounts, and in some peels was exceeded only by oxalate. Malic and citric acids were also determined in each peel. At least 15 peaks appeared with each peel, and paper chromatography of the effluents indicated the presence of more than one component in several peaks.

The chemistry of citrus fruits and changes associated with maturation have been studied by many workers. This information has been summarized in recent reviews (Kefford, 1959; Sinclair, 1961). More recently, the author and co-workers have presented data regarding free amino acids in citrus fruits and the variations which accompany maturation (Clements and Leland, 1962a,b). This report is an extension of these studies to include the nonvolatile organic acids in peel and juice of different varieties, preliminary to investigations of maturation changes.

Knowledge regarding the citrus acids and the role they play in determining the acidity and buffering properties of the fruit is summarized in the aforementioned reviews (Kefford, 1959; Sinclair, 1961). In general, citric acid predominates in the flesh of the fruits, and oxalate predominates in the peels; several other acids have been reported in variable amounts in the different varieties. Citric acid has received much attention because of its obvious contribution to the properties of the juices of the commercial varieties, but quantitative data on the "minor" acids of the fruits are relatively limited. Such acids, as intermediates in the metabolic processes of the fruit, are directly involved in growth, maturation, and senescence. The present study was undertaken to determine differences among varieties, primarily to provide a basis for future studies correlating chemical composition with maturation behavior. Such information should also be of value in interpreting postharvest characteristics and taxonomic relationships.

The procedure of Bulen *et al.* (1952) was modified to serve as a method of analysis. For the juices, direct addition of the freeze-dried solids to the silicic-acid column was satisfactory. For peel studies, however, this technique resulted in poor resolution and low recoveries. Ether-extraction of the freeze-dried peel also presented several difficulties. Prolonged extraction (several days) was required for complete extraction, and the large amounts of sulfuric acid required to liberate oxalate from its calcium salt resulted in a moist product unsatisfactory for ether extraction without addition of adsorbent. Although such procedures have been applied in citrus peels (Sinclair and Eny, 1947), analysis of such extracts on silicic-acid columns gave erratic results and produced peaks which appeared to be artifacts.

Cation-exchange columns in the hydrogen form are used extensively for the acidification of liquids. Bradley (1960) used such

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a procedure for studies of tomato purée, and eliminated sulfuric acid from the analytical silicic-acid column. This technique made it possible to determine the slower-moving acids (including the mineral acids), the latter being eluted with methyl alcohol. It was concluded from this report that an analagous procedure might be used for acidification and extraction of acids from solids for consequent application to the conventional silicic-acid column. Treatment with cation-exchange resin in the hydrogen form is less drastic than addition of relatively concentrated sulfuric acid to the dry material. It also permits extraction with a more polar solvent (and therefore one in which most of the acids are more soluble), since the addition of extractable acid is avoided. Such an extract, free of extraneous acid, provides a measure of total-anion content. This technique was applied to peels in the following study, and should be applicable to other materials.

EXPERIMENTAL

Source of fruit. Arizona and Texas grapefruits and one sample of Washington Navel orange (Sample III) were obtained from local markets. All other samples were picked from trees in plots of the Citrus Research Center at Riverside, California.

Preparation of fruit. Each sample (ten fruit) was washed and weighed. Pulps were pooled, weighed, sliced, and crushed in a mortar. Juice was expressed by squeezing through cheesecloth, and was finally vacuum-filtered through Celite (J-M Filter-Aid) to give a clear serum. Solids, pH, and total acid were determined, and the serum was freeze-dried and stored over calcium chloride. Peels were pooled and weighed, and ground in a homogenizer (Waring blender) without addition of water. The homogenate was frozen in a polyethylene bag, and several such samples were freeze-dried simultaneously in the apparatus previously described (Clements, 1962). The dry peel was stored over calcium chloride.

Extraction of acids from peel. Freeze-dried peel (1 g) was ground finely in a mortar with an equal weight of dry Amberlite IR-120 resin (Rohm and Haas Co.), converted to the hydrogen form as described by Moore *et al.* (1958), washed free of chloride, and dried at 60°C. The mixture was transferred to a wide-mouth screw-cap vial, and water was mixed in to give a smooth paste. The vial was capped and stored 4–5 days at 4°C. Silicic acid (washed and dried) was then mixed with the sample to give a free-flowing powder, and the mixture was slurried in acid-free methanol and transferred to a glass column (22 mm ID × 20 cm) equipped with a stopcock at the lower end, and with a glass-wool plug above the stopcock. A reservoir (200 ml), attached to the top of the column with a 19/38 ℉ joint and fitted with a squeeze bulb, facilitated the application of pressure. The liquid was forced out until level with the top of the solids, and additional methanol was forced through the column until the effluent was acid-free. The combined effluent (50–60 ml) was titrated with 0.2*N* sodium hydroxide, with phenol red as an indicator, and evaporated to dryness in an air stream. The residue was analyzed immediately or stored at −4°C.

Determination of acids. The following modification of Bulen *et al.* (1952) was used: Silicic acid (10 g), washed free of fines and dried, was ground with 0.5*N* sulfuric acid (7.2 ml). The mixture was slurried in washed chloroform and packed in a column (12 mm ID × 35 cm). The sample (50 mg juice solids or extract of 1 g peel) was acidified with 5*N* sulfuric acid (0.5 ml), taken up with a minimum amount of silicic acid, and added to the top of the column. The acids eluted with mixtures of *n*-butanol in chloroform equilibrated against 0.5*N* sulfuric acid according to the following schedule: 75 ml each of 2.5, 5, 10, 15, 20, and 25%, followed by 250 ml of 35% (butanol in chloroform, v/v). Fractions (3.3 ml) were collected, and titrated with 0.01*N* sodium hydroxide (aqueous), with phenol red as indicator. The flow rate was approximately 100 ml per hour.

Paper chromatography. Peaks were tentatively identified by comparison of elution thresholds with known compounds. For further identification, fractions representing individual peaks were combined, evaporated, and chromatographed on paper. Two dissimilar solvent systems were used: I) *i*-amyl alcohol equilibrated against 5*M* formic acid (Clements and Deatherage, 1957); and II) pyridine-ethyl acetate–acetic acid–water (5:5:1:3, v/v/v/v) (Fischer and Dorfel, 1955). Small-scale one-dimensional chromatograms were run as previously described (Clements and Deatherage, 1957) on S & S 589 White Ribbon acid-washed paper. R_f values were measured, but in every case unknowns were checked against known compounds on the same chromatogram. Spots were detected by UV fluorescence and adsorption, bromophenol blue, and ammoniacal silver nitrate (Buch *et al.*, 1952). Color development with the last reagent was followed for several days, since it appears to be characteristic for individual compounds. Only those peaks which gave positive reactions with bromophenol blue on the chromatograms are reported quantitatively, however.

RESULTS AND DISCUSSION

Evaluation of methods. Fig. 1 shows elution diagrams for a calibration column and for an analysis of a resin-treated lemon peel. Peak positions were determined for several other known acids, but the standard separation (which is illustrated) was primarily for the purpose of determining recoveries of those acids found to occur in citrus peels. These recoveries were determined by subjecting weighed amounts of acids to a resin treatment and extraction identical to the procedure used for peel analysis. Recoveries were comparable to those reported by Bulen *et al.* (1952) (i.e., 95–100% for most acids). Separate studies were performed on calcium oxalate, to determine the rate of solubilization. Results indicated that free oxalate was released from the relatively insoluble salt almost immediately. Recoveries exceeded those reported by Bulen *et al.* (85%). None of the acids studied appeared to be altered when subjected to contact with the resin (aqueous suspension) for several days at 25°C.

The period (4–5 days) used for peel treatment was determined by experiment,

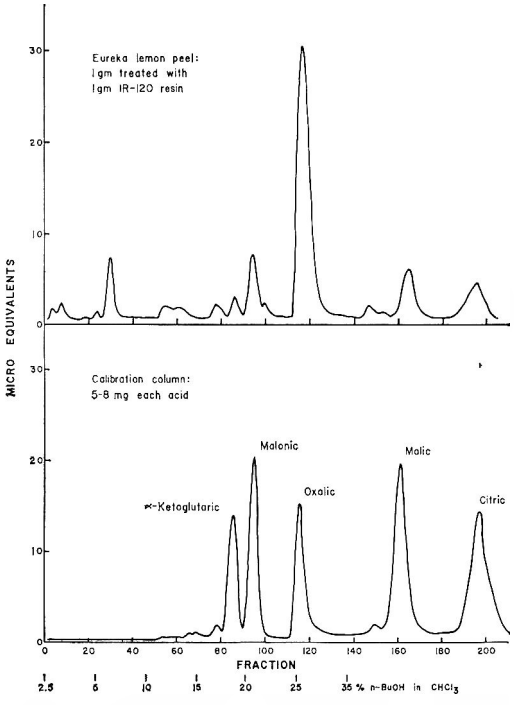

Fig. 1. Organic acids from lemon peel acidified with cation-exchange resin in the hydrogen form.

and appeared to be sufficient for equilibration (as evidenced by chromatograms from various treatments). The procedure produced sharp, reproducible separations, and appears to be especially advantageous for determinations of oxalate when it is present as the calcium salt. Total-anion values, determined by direct titration of the extract from duplicate samples, consistently agreed within 3–4%.

Compositions of juices. Data regarding the juices are presented in Table 1, and for those varieties which have been studied by other workers, the compositions are generally within the ranges reported (Sinclair, 1961; Wolf, 1958). Characteristically, citric acid predominated and malic acid appeared at low, but significant, levels. However, an exception was the Palestine sweet lime. The solids-acid ratio of the sample was 99 (compared to 12–13 for the oranges); the pH was 5.7, considerably higher than that of any other variety. The fruit which were analyzed displayed physical characteristics indicative of a physiological stage comparable to that of oranges during commercial maturity (i.e., with respect to solids content of juice, moisture content of peel, degree of turgidity, etc.). It is interesting to note that this was the only variety in which malic acid predominated in the juice (although malate concentration was comparable to that in the other varieties). The data suggest that the degree of acidity which characterizes a given variety is almost entirely a function of citrate content.

Silicic-acid separations of the various juices indicated that acids in addition to malate and citrate were present in the effluent, though at extremely low levels. Ascorbic acid, galacturonic acid, and other slow-moving acids would not appear in the effluent. Ting and Deszyck (1959) reported *l*-quinic acid in the peel and pulp of Pineapple oranges. Elution of this acid from a silicic-acid column requires the addition of 50% *n*-butanol in chloroform (following 35%). This sequence was followed on single samples of peels and juices of the varieties in preliminary stages of this study, but additional measurable peaks did not appear. However, sensitivity is reduced with this concentration of butanol (because of high

Table 1. Citric and malic acid contents of juices of some citrus varieties.

Variety	Solids (%)	Acid (as citric) (%)	Solids acid	pH	Meq per 100 mg solids: Malic acid	Meq per 100 mg solids: Citric acid	Grams per 100 ml juice: Malic acid	Grams per 100 ml juice: Citric acid
Orange								
Wash. Navel (I)	6.8	0.55	12.4	3.6	13	129	0.06	0.56
Wash. Navel (II)	6.4	.51	12.5	3.6	11	132	.05	.54
Wash. Navel (III)	12.7	.95	13.4	3.6	23	115	.20	.93
Valencia	12.9	1.04	12.4	3.4	19	119	.16	.98
Grapefruit								
Calif. (Marsh)	10.1	1.80	5.6	2.9	9	276	.06	1.79
Arizona	11.6	1.98	5.9	3.1	5	284	.04	2.10
Texas (pink)	9.3	1.14	8.2	3.3	9	201	.06	1.19
Lemon								
Eureka (I)	8.6	4.40	2.0	2.5	29	726	.17	4.00
Eureka (II)	8.6	4.57	1.9	2.4	46	796	.26	4.38
Lime								
Palestine Sweet	9.9	.10	99.0	5.7	30	1	.20	.08
Tangerine								
Dancy (I)	11.7	1.00	11.7	3.5	23	164	.18	1.22
Dancy (II)	11.3	.84	13.5	3.6	28	119	.21	.86

background and broad peaks), and quinic acid must be present in substantial amounts to produce a distinct peak.

Composition of peels. Characteristics of the peels are presented in Table 2. In general, the varieties displayed elution patterns qualitatively similar to that of the lemon (Fig. 1). Paper chromatography of column effluents indicated that most of the peaks contained more than one compound detectable with ammoniacal silver nitrate. However, the predominant acids (oxalic, malic, malonic, and citric) produced peaks which appeared to be relatively pure. These acids also appeared at levels sufficient to give a strong positive reaction with bromophenol blue when the peaks were chromatographed on paper. The fractions containing malonic acid consistently contained another compound which exhibited R_f values and color

Table 2. Organic acids in the peel of some citrus varieties.

Variety	Weight (g) (av. per fruit): Fruit	Pulp	Peel: Wet	Dry	Meq. per g dry weight of peel: Total anion extd.	Citric acid	Malic acid	Oxalic acid	Malonic acid
Orange									
Wash. Navel (I)	145.9	99.0	41.5	11.8	0.40	0.01	0.02	0.11	0.02
Wash. Navel (II)	145.1	101.2	40.5	11.5	.41	.01	.02	.12	.01
Wash. Navel (III)	143.3	102.8	36.1	10.3	.41	.01	.02	.10	.03
Valencia	115.8	88.2	26.1	7.7	.44	Trace	.02	.13	.03
Grapefruit									
Calif. (Marsh)	286.2	193.3	90.5	20.3	.32	.01	.03	.06	.02
Arizona	361.1	243.7	112.8	28.3	.59	.03	.10	.12	.02
Texas (pink)	395.4	311.7	80.2	18.8	.53	.01	.08	.08	.02
Lemon									
Eureka (I)	97.8	69.2	27.0	5.5	.60	.04	.04	.15	.03
Eureka (II)	92.5	64.5	26.7	5.5	.53	.03	.02	.12	.04
Lime									
Palestine Sweet	137.7	115.7	21.5	4.9	.46	Trace	.04	.05	.05
Tangerine									
Dancy (I)	108.2	81.7	25.0	6.9	.60	.02	.06	.15	.01
Dancy (II)	70.2	48.6	20.9	5.4	.68	.02	.09	.20	.02

reactions characteristic of chlorogenic acid. However, it did not appear in peel effluents in sufficient amounts to give an acid reaction with bromophenol blue on paper chromatograms. The oxalate fractions also contained other compounds, though in relatively small amounts. Each of the peels produced at least fifteen different peaks, and a total of 25–30 components on paper chromatograms. No attempts were made to retain volatile acids, and, as with the juices, the slow-moving acids would not appear in the column effluent.

Table 2 shows that oxalate predominated in every peel except that of the Texas grapefruit, in which oxalate and malate appeared in equivalent amounts; malate content approached that of oxalate in the other grapefruit. The lemons and tangerine exhibited the highest total-anion and oxalate contents. Subsequent data (in preparation) show that the peel acids occur in considerably higher concentrations in flavedo than in albedo (approximately 2:1). Therefore, high acid content in tangerine peel may be a reflection of low albedo content in this fruit. It also suggests that lemon flavedo would demonstrate a relatively high acid content.

The values for oxalate, citrate, and malate in peels of orange, lemon, and grapefruit agree closely with those reported by Sinclair and Eny (1947). Those authors did not report malonate, although Biale and Young (1962) found malonic acid in lemon peel. In the present study, malonic acid content was exceeded only by that of oxalate (in several instances); malonate and oxalate appeared in equivalent amounts in the sample of Palestine sweet lime. Hatch and Stumpf (1962) reviewed the occurrence and possible roles of malonic acid in plants, and concluded from their own experiments that enzymes for the synthesis and breakdown of malonate are widely distributed among plants. Although the present study involved relatively limited sampling, it indicates that malonic acid is normally present in measurable amounts in the peels of mature citrus fruits. It does not appear to be present in the pulp, however. The role of malonate, as well as that of the other carboxylic acids which accumulate in these fruits, remains to be determined.

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Organic Acids in Citrus Fruits. II. Seasonal Changes in the Orange[a]

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SUMMARY

Organic acids were quantitatively determined by silicic-acid chromatography in juice of Valencia oranges sampled from trees over a 16-month period, and in the juice, albedo, and flavedo of Washington Navel oranges sampled over a 12-month period. Results obtained prior to late maturity generally agreed with existing data, but previously unreported changes occurred during and after late maturity. In the juices, citrate predominated, but declined on a per-fruit basis in late season; malate appeared at a low level. In the Navel flavedo and albedo, oxalate decreased and malonate increased. Through most of the season, albedo acid composition was comparable to that of diluted flavedo, but in the latest sample, malonate predominated in the flavedo and oxalate predominated in the albedo. Malate appeared in low, decreasing amounts in the peel, with lesser amounts of citrate. These four acids accounted for 30–50% of the total anions in the peel fractions.

The present paper reports on a continuation of investigations of chemical changes related to maturation and senescence in the citrus fruits. A previous report (Clements, 1964) presented quantitative data regarding organic acids in the juices and peels of several citrus varieties. This study substantiated published reports that juices of mature citrus fruits are generally characterized by a predominance of citric acid. Malic acid occurred at a rather uniform, low level in all of the varieties studied, and appeared to be independent of citrate concentration. Data regarding the peels indicated that oxalate generally predominated, with variable amounts of malate and traces of citrate occurring. However, in addition to these previously reported acids, malonic acid was detected in variable amounts in each of the varieties, and in concentrations equivalent to oxalate in one variety (Palestine sweet lime).

In view of the role of organic acids in the metabolic processes of the fruit, and the importance of the acids in determining the character of the edible portion, this seasonal study was performed. The data present quantitative measurements of the predominant acids in the juice of the Valencia orange over a 16-month period, and in the juice, albedo, and flavedo of the Washington Navel orange during a period of approximately 12 months.

EXPERIMENTAL

Sources of fruit. The Valencia oranges were samples that had served as a basis for a previous investigation of seasonal changes in free amino acids (Clements and Leland, 1962), and analyses were performed on portions of the same juices. The fruit was picked at approximately monthly intervals from trees in plots of the Citrus Research Center at Riverside, California, embracing the period from September 30, 1959, to December 12, 1960. The Washington Navel oranges were also from Citrus Research Center plots. The sampling procedure was similar to that described for the Valencia fruit except that five trees were sampled at approximately monthly intervals from August 2, 1960, to July 18, 1961. Each sampling consisted of three lots of ten fruit, each lot consisting of two fruits from each tree.

Sample preparation. Juice from the Valencia oranges was expressed, filtered, and freeze-dried as previously described (Clements and Leland, 1962); the peel was discarded. The Navel oranges

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Table 1. Seasonal changes in some gross measurements of the Washington Naval orange.[a]

	Diam. (mm)	Weight (g)						Juice (ml)
				Albedo		Flavedo		
		Fruit	Pulp	Wet	Dry	Wet	Dry	
Aug. 2, '60	47	51.5	25.1	13.3	3.8	12.2	3.6	8.2
Sept. 12	56	90.6	58.9	14.1	3.9	15.3	4.4	30.0
Oct. 19	63	130.9	88.1	20.3	5.2	17.4	4.4	48.5
Nov. 15	65	143.5	96.3	19.7	5.3	20.5	5.4	50.8
Dec. 12	66	145.5	100.1	18.5	5.4	22.6	6.3	50.8
Jan. 10, '61	66	145.6	101.4	18.7	5.8	20.9	6.2	51.5
Feb. 20	65	144.8	98.9	18.3	5.6	21.0	6.0	50.0
Apr. 3	63	132.8	91.4	17.4	4.9	18.5	4.9	54.0
May 15	62	129.1	90.2	16.6	4.8	18.0	5.0	51.3
July 18	62	129.6	92.7	18.2	4.8	14.7	5.4	54.0

[a] Averages for individual fruits, derived from measurements on duplicate lots of ten fruit each.

were processed in a similar manner, but albedo and flavedo were separated and freeze-dried by the procedure outlined for peel preparation in the varietal study (Clements, 1964). Of the three lots obtained in each sampling, the two which agreed most closely with respect to juice characteristics (solids content, acidity, and pH) were utilized for analysis, and the data represent averages of each of these sets of duplicates. The third lot was not used. This technique was adopted to minimize variation introduced by inclusion of "off-bloom" fruit.

Analytical procedure. Acids in the juices were determined by silicic-acid chromatography of the freeze-dried serum by a modification of the procedure of Bulen *et al.* (1952). Since the only acids detected in measurable amounts were citric and malic, a short column and single eluting solvent were utilized. The column was prepared by suspending 7 g silicic acid (acidified with 4.6 ml 0.5*N* sulfuric acid) in chloroform saturated with 0.5*N* sulfuric acid, and packing in a tube 12 mm ID. Freeze-dried serum (50 mg) was applied to the column, and citric and malic acids were eluted with 35% *n*-butanol in chloroform (approximately 250 ml). Acids in Navel albedo and flavedo were extracted and determined as described in the varietal study (Clements, 1964). Results represent averages of duplicate determinations performed on duplicate samples.

RESULTS AND DISCUSSION

Gross measurements of the Washington Navel orange are presented in Table 1. Measurements of the Valencia samples have been published (Clements and Leland, 1962). Table 2 shows variations in the juice properties of the two varieties. The Valencia study covered a period of approximately 14 months, starting on September 30, 1959. However, a single sample of twelve fruit was picked from the same trees two months prior to this, and although no gross measurements were made, an acid analysis was performed and the results are included in Fig. 1.

Table 2. Seasonal variations in juices of Valencia and Washington Navel oranges.

Date	Solids (%)	Acid (as citric) (%)	Solids acid	pH
Valencia				
Sept. 30, '59	7.9	2.6	3.1	2.9
Nov. 2	8.4	2.5	3.4	2.9
Dec. 1	9.5	2.2	4.3	2.9
Jan. 15, '60	10.2	1.9	5.5	3.0
Feb. 24	11.0	1.5	7.6	3.1
Mar. 16	11.5	1.4	8.5	3.2
Apr. 4	12.1	1.3	9.5	3.3
May 4	12.4	1.2	10.0	3.4
June 1	12.8	1.0	12.4	3.5
July 5	12.0	.8	14.7	3.6
Aug. 29	12.8	.7	19.6	4.0
Oct. 5	13.4	.6	24.4	4.1
Nov. 4	15.3	.6	25.8	4.1
Dec. 12	16.5	.6	26.5	4.1
Washington Navel				
Aug. 2, '60	10.1	2.1	4.8	3.1
Sept. 12	9.1	1.8	5.1	3.0
Oct. 19	9.5	1.0	9.5	3.4
Nov. 15	6.4	.6	10.7	3.5
Dec. 12	6.6	.5	13.2	3.6
Jan. 10, '61	9.1	.6	15.2	3.6
Feb. 20	14.2	.9	15.8	3.7
Apr. 3	14.4	.7	20.6	3.9
May 15	15.2	.7	21.7	3.9
July 18	16.5	.6	27.5	4.5

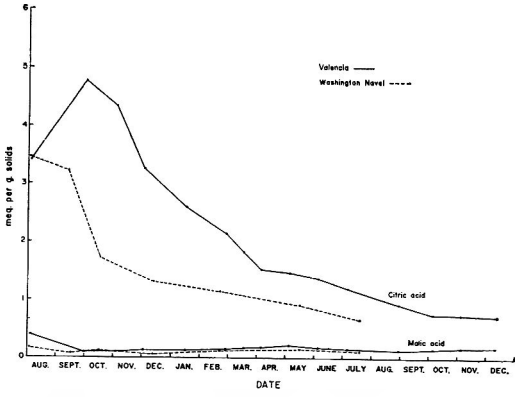

Fig. 1. Seasonal variations in acids in the juices of Valencia and Washington Navel oranges.

The Navel study involved ten samplings, starting on August 2, 1960, and covering slightly less than twelve months; acid analyses were carried out on seven of these samplings. In general, pertinent investigations by previous workers covered much shorter periods, terminating at or before late commercial maturity. In the present study, both varieties of oranges were sampled beyond commercially acceptable stages. The final samplings appeared to be physiologically sound, but were organoleptically unacceptable (primarily because of low acid and a benzaldehyde-like off-flavor).

The data in Tables 1 and 2 pertaining to the Navel orange present a picture which appears to be characteristic of fruit which has been allowed to remain on the tree beyond maturity. Maximum size was attained in December-January, after market maturity (October), but then declined. Most of the loss can be accounted for by dehydration of the pulp and flavedo, but there was also a loss in dry weight in both albedo and flavedo. Free acid in the Valencia juice decreased steadily until near the end of the sampling period, but in the Navel fruit it reached a minimum and then rose slightly.

Juice acids. Seasonal changes in citric and malic acids in the juices of the two varieties are shown graphically in Fig. 1. All analyses were performed on the juice solids, and the results are presented on this basis. The citric-acid curves for the fruits show a general similarity, demonstrating a rapid decline prior to maturity (due to dilution by other solids). A more gradual decrease occurred during the periods of maturity, and a lesser, but steady, decline continued until the end of each sampling period. The decrease in citrate in the Valencia fruit was more gradual than that of the Navel, and displaced 4–5 months later. By coincidence, one sampling of each variety exhibited a solids-acid value of 9.5, a value representative of the early stages of maturity. This value was attained on April 4 by the Valencia orange, and on October 19 by the Navel, and these dates occupy similar positions on the respective citric-acid curves (Fig. 1). Because of the relatively earlier beginning of the Valencia sampling, a maximum in the citrate curve appeared during the first two months. It is assumed that a similar peak would have been demonstrated by the Navel fruit if sampling had been started earlier (Sinclair, 1961).

Malic-acid curves for the two varieties almost coincide (Fig. 1). On a solids basis, malate concentrations remained relatively constant throughout the season, generally being present in amounts of 0.1–0.2 meq per g juice solids. In the late fruit, malate constituted approximately 20% of the total acid in each variety.

Peel acids. Seasonal variations in the flavedo acids of the Washington Navel orange are best illustrated by elution diagrams of some individual analyses (Fig. 2); data regarding those acids which were determined quantitatively are summarized in Table 3. In general, total-anion content of the flavedo was almost twice that of the albedo, and this was reflected in the levels of the individual acids. Citric, malic, oxalic, and malonic acids accounted for approximately 35% of the total anions in the early albedo, but for only 30% in the late albedo. In the early flavedo, these acids made up slightly more than 35% of the total anions, but this proportion rose to approximately 50% in the late fruit. In both albedo and flavedo, total-anion values decreased in early season to a minimum during maturity (December), and then increased.

The most significant changes during the sampling period were in levels of oxalic and malonic acids. Oxalate predominated in both albedo and flavedo in the early fruit, while malonate contributed less than 0.01 meq

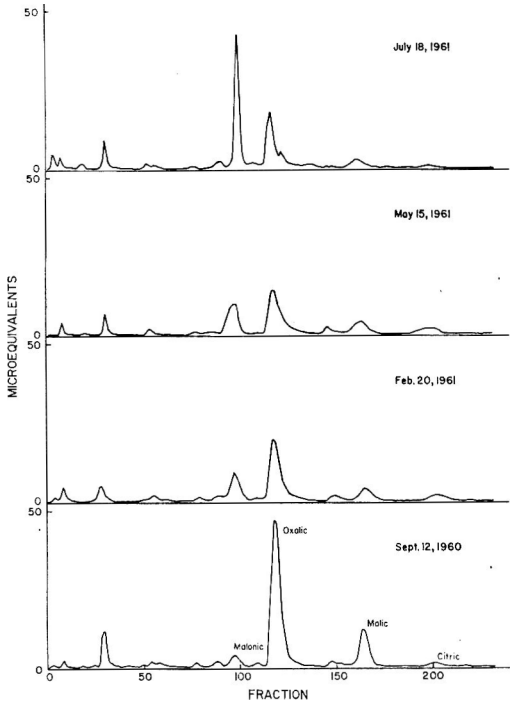

Fig. 2. Seasonal changes in flavedo acids of the Washington Navel orange as reflected in silicic-acid column elution patterns (1 g dry flavedo, analyzed as described in text).

per g dry weight in either tissue. However, oxalate declined quite steadily (from 0.14 to 0.06 meq per g in albedo, and from 0.23 to 0.10 meq per g in flavedo), and malonate increased (from traces to 0.03 meq per g in albedo, and from 0.01 to 0.12 meq per g in flavedo). It is noteworthy that through most of the season albedo acid composition was a reflection of flavedo composition (i.e., albedo behaved as though it were diluted flavedo). However, in the final samples, the malonate increase and oxalate decrease in the flavedo had resulted in a predominance of malonate in the flavedo, whereas oxalate still predominated in the albedo.

Citric acid appeared in small amounts in both albedo and flavedo, attaining a level of 0.01–0.02 meq per g in the late flavedo. Malic acid was present in higher levels (0.01–0.03 meq per g in albedo, and 0.03–0.07 meq per g in flavedo), and exhibited a general decrease during the sampling period.

Variations on a per-fruit basis. In a study such as this, in which trends are of particular interest, calculations of absolute amounts of

Table 3. Seasonal changes in the organic acids in the peel of the Washington Navel orange.

Date	Meq per g dry weight									
	Albedo					Flavedo				
	Total anion extd.	Citric acid	Malic acid	Oxalic acid	Malonic acid	Total anion extd.	Citric acid	Malic acid	Oxalic acid	Malonic acid
Aug. 2, 1960	0.50	0.01	0.03	0.14	Trace	0.81	Trace	0.06	0.23	0.01
Sept. 12	.44	Trace	.03	.13	Trace	.77	Trace	.07	.29	.03
Oct. 19.	.38	Trace	.02	.09	.01	.68	Trace	.05	.20	.03
Dec. 12	.30	Trace	.01	.09	.01	.51	.01	.04	.15	.02
Feb. 20, 1961	.32	Trace	.01	.09	.02	.54	.02	.03	.15	.05
May 15	.30	.01	.02	.05	.02	.56	.02	.04	.12	.08
July 18	.40	.01	.02	.06	.03	.64	.01	.03	.10	.12

individual components per fruit are of value. Such data provide balance information regarding net losses or gains, and eliminate variations which are merely dilution effects. In this study, insoluble solids in the pulp were not determined, and exact per-fruit values cannot be calculated for juice components. However, approximate values for a soluble component can be derived by multiplying the dry-weight concentration of the component by the product of pulp weight and solids concentration in the juice. This approximation does not allow for weight of water-insoluble material (less than 5% of wet weight of the mature pulp), and assumes that juice which remains in the pulp residue is identical to that expressed. The error would be expected to vary during the season, but it was concluded that the data would be sufficiently accurate for purposes of comparison, particularly in the mature and post-mature stages.

In the Valencia orange, citric acid in the pulp attained a maximum of approximately 26 meq per fruit in November, and gradually declined to less than 20 meq per fruit at early maturity (March). This value remained relatively constant until June. This agrees very closely with results reported by Sinclair and Ramsey (1944) for the maturation phase, with respect to both level of acid and lack of change. However, as the fruit remained on the tree beyond maturity, citrate steadily declined to 9 meq per fruit (December), less than half the level at maturity. This post-maturity behavior is in contrast to observations preceding and during maturation which would indicate that decreasing acidity during growth is solely a dilution effect (Sinclair, 1961). A similar decline was noted in the Navel pulp after maturity (from 16 meq per fruit in February to 10 meq per fruit in July). Apparently, citrate translocation and/or conversion exceed accumulation at this stage.

Per-fruit data for the Navel peel were calculated from the dry-weight values of albedo and flavedo. Total-anion content in the flavedo remained relatively constant during the entire sampling period, ranging from 2.8 to 3.5 meq per fruit. In the albedo, total anion ranged from 1.4 to 2.7 meq per fruit, with no definite trend appearing. Malate remained at a constant low level in both albedo (0.1 meq per fruit) and flavedo (0.2 meq per fruit). The changes in oxalate and malonate were still pronounced when calculated per fruit. Oxalate decreased slightly in albedo after maturity, from 0.5 to 0.2 meq per fruit, but the decline was more evident in the flavedo. After an initial rise in the early fruit (from 0.8 to 1.3 meq per fruit during August–September), it steadily decreased to 0.5 meq per fruit in the last sampling (July). Malonate accumulated to approximately 0.1 meq per fruit in the albedo, but increased almost 16-fold to 0.7 meq per fruit in the flavedo. Most of this accumulation occurred after maturity.

Conclusions. For the most part, data from the study agree with that collected by previous workers during pre-maturation and maturation periods. However, this study has provided new information regarding late- and post-maturity stages, and in the case of the Navel orange, has provided new data concerning differences in albedo and flavedo fractions of the peel. The results are based on limited sampling, but the frequency of sampling and accuracy of methods have demonstrated some definite trends in the fruits which were studied. These trends cannot be extrapolated to other varieties of citrus, or even to the same varieties grown under other conditions, but they suggest some metabolic transitions which can serve as bases for further investigation.

Among the most important observations were the decrease in oxalate and the increase in malonate in the peel of the Washington Navel orange, particularly after maturity. The biochemistry of these acids in plants has been reviewed by Crombie (1960), and previous investigations of these "extracyclic" acids permit some speculation concerning their formation and disappearance. No definite conclusions can be drawn regarding their roles in citrus, however. Hulme (1958) has suggested that the breakdown of some fruits may be the result of accumulation of respiratory inhibitors, and malonic acid build-up is of interest in this respect. Hatch and Stumpf (1962) reviewed the occurrence of malonate in plants, and their studies suggest that malonate is widely distributed in the plant kingdom. Although

the evidence indicates that oxalate decrease and malonate increase in Naval peel (as well as citrate disappearance in the juices) occurred primarily after commercial maturity, these same processes may be active earlier in the fruit. The variations may represent net effects resulting from shifts which occur during the later stages of fruit development.

It would be of interest to determine whether the seasonal variations which have been detected in this study are typical of citrus fruits in general, and whether such changes occur in the detached fruit. Such information would be of value in determining the factors which influence keeping quality. It would be of particular value in delineating the biochemistry and physiology of maturation and senescence.

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The Organic Constituents of Food. III. Sweet Potato

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INTRODUCTION

In 1962, the sweet potato *(Ipomoea batatas)* (Convolvulaceae) ranked seventh by weight in per capita consumption in the United States. The vegetable is particularly popular in the Southern states, where it was introduced into this country and has been known as far back as 1648. However, world production figures show that its use in the United States is very small in comparison to that in other countries. Total world production for 1961 was some 108,300,000 metric tons. Of this, the United States produced 701,000 tons, China (Taiwan) 2,979,000 tons, Indonesia 2,709,000 tons, and Japan, which led world production, 6,277,000 metric tons. The reason for its great popularity in these countries is readily apparent in light of their present food shortages—the sweet potato is second only to epos root and corn in calorie content, and so represents an inexpensive, concentrated source of food energy.

The sweet potato was originally called the "Spanish potato," to distinguish it from the Irish potato *(Solanum tuberosum)* (Solanaceae). (The word "potato" appears to have been derived from the Indian word for sweet potato, *batata*.) In the southern part of the United States, the sweet potato is generally known as "yam," although it has no botanical relation to the true yam *(Dioscorea sativa)* which may actually be poisonous. *Ipomoea batatas* probably had its origin as a food in ancient Central America with the discovery that the narrow roots of certain morning glory plants were edible. The cultivation of these plants eventually created tubers that were quite large, the normal weight being several pounds each. Gradually, use of the plant spread to Mexico, South America, North America, and Europe. An interesting history of the sweet potato is given by Quinn (1942).

Today, numerous varieties of the sweet potato are used for food, although by far the major use actually lies in cattle feed. The most important commercial varieties used for food are the Puerto Rico and the Jersey; others are the Nancy Hall, Red Nancy, All Gold, and Rols. Of the "feed" varieties, the Pelican Processor and the Whitestar are the most common.

COMPOSITION

Macromolecular constituents. Recent data on the proximate composition of the edible portion of the sweet potato are presented in Table 1. The water content (which also includes all easily volatile constituents such as simple alcohols, amines, aldehydes, etc.) is strikingly low—in fact, among the lowest of any common vegetable. This makes the tuber an exceptionally concentrated source of food as well as of the minor chemical constituents.

Table 1. Proximate composition of sweet potato (Howard *et al.*, 1952).

Variety	Water	Protein	Fat	Total sugar	Other carbohydrates
	Percent of fresh weight				
Jersey	67	2.0	0.2	6.0	21.0
Puerto Rico	70	2.0	0.2	4.4	.....
Velvet	69	1.4	0.2	5.8	16.3

Despite this, the protein content is not unusual. The total amino acid composition has been summarized (Orr and Watt, 1957) (Table 2), and the composition of isolated protein has been reported with respect to several specific amino acids (Nishi, 1949).

Particular attention has been paid to the globulin fraction from sweet potato. The greater part of

Table 2. Total amino acid composition of sweet potato (Orr and Watt, 1957).

Amino acid	g/100 g ed. portion	Amino acid	g/100 g ed. portion
Arginine	.094	Methionine	.033
Cystine	.029	Phenylalanine	.100
Glutamic acid	.334	Serine	.046
Histidine	.036	Threonine	.085
Isoleucine	.087	Tryptophan	.031
Leucine	.103	Tyrosine	.081
Lysine	.085	Valine	.135

the protein—perhaps most of it—is represented by a specific globulin, "ipomoein" (Jones and Gersdorff, 1931). Upon aging of the tuber in storage, ipomoein is converted by the action of proteolytic enzymes to a polypeptide differing considerably from the parent globulin in its physical and chemical properties.

With the possible exception of the epos root (*Perideridia oregana*) (Umbelliferae), consumed by Northern California Indians, sweet potato appears to have the highest non-sugar carbohydrate content of any vegetable food. Matsuo and Namba (1959) presented information on the celluloses, hemicelluloses, starch, pentosans, and other high-molecular-weight carbohydrates, but that account is not generally available. Williams *et al.* (1940) offered some data on the same subject, and the content of starch, pectic substances, and other types of carbohydrate has been investigated by many workers (Scharpenseel, 1957; Elwell and Dehn, 1939; Baba and Shimabayashi, 1952a,b).

The acid hydrolysate of sweet potato starch which has undergone extensive reversion yielded two interesting disaccharides: "α-kojihiose" and "β-kojibiose" (Sato and Aso, 1957). These reducing sugars, isolated as their octaacetates, were shown to be epimers of 2-*O*-α-D-glucopyranosyl-D-glucose by comparison with synthetic samples (Matsuda, 1957).

The aromatic polymers, such as tannins, lignins, and polyphenols, have received superficial attention (Matsuo and Namba, 1959; Nishida *et al.*, 1952; Uritani, 1953), particularly with respect to the changes which occur in diseased tubers. However, other macromolecular constituents such as nucleic acids and polyterpenes appear not to have been investigated.

Common constituents of low molecular weight. Although a few of the common sugars have been identified in sweet potatoes (Table 3), a search of the literature failed to uncover evidence for the nature of the "fat" indicated from the proximate analysis. Interestingly enough, Eckey (1954) listed and compared the fats of other members of the Convolvulaceae—generally oils containing the usual palmitic, stearic, C_{20}–C_{22}, oleic, linoleic, and linolenic acids—but no mention was made of *Ipomoea batatas*.

A few of the other anticipated common acids have been reported (Table 3), but their identity has seldom been confirmed by isolation.

The vitamins detected in sweet potato are listed in Table 4. The tuber is a rather good general source and is exceptional in the case of vitamin A. The free amino acids appear not to have been determined.

Table 4. The vitamins of sweet potato (Burton, 1959; Sebrell and Harris, 1954).

Vitamin	μg/100 g	Vitamin	μg/100 g
A	2,310	Niacin	600
B_1 (thiamine)	90	Pantothenic acid	940
B_2	50	Biotin	43
B_6 (pyridoxine)	320	Choline	350
C (ascorbic acid)	22,000	*p*-Aminobenzoic acid	6–12
E	4,000	Folic acid	5–19

Flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) have been reported (Mitsuda *et al.*, 1958) and, of course, are to be expected. It appears that the proportions of FAD and riboflavin are approximately equal, whereas FMN makes up only about 2% of the total flavins.

Unusual or specific constituents. Of the almost innumerable aliphatic compounds probably present in *Ipomoea batatas*, only acetoin and methylglyoxal (pyruvaldehyde) have been reported (Hanai, 1956), and these only as the oximes on paper chromatograms from extracts of the cooked or fermented vegetable. However, the bright orange

Table 3. Common constituents of sweet potato.

	Evidence	Source	Reference
Sugars			
Glucose	Isolation [a]	Tubers	Miyake, 1915
Fructose	Color tests	Tubers	Miyake, 1915
Sucrose	Isolation	Tubers	Miyake, 1915
Organic acids			
Citric acid	Analysis	Tubers	Hartmann and Hillig, 1934
Oxalic acid	Isolation [b]	Tubers	Andrews and Viser, 1951
Malic acid	Analysis	Tubers	Prokoshev and Minina, 1954
Tartaric acid	Paper chromatography	Tubers	Hayashi and Takijima, 1956

[a] Isolated as phenylglucosazone.
[b] Isolated as calcium oxalate.

color of the tuber has led to many investigations of the carotenoid content. Matlack (1937), using column chromatography, was able to separate four colored constituents from sweet potato extracts. Only one was obtained in crystalline form, and this was identified as β-carotene. Ultraviolet spectra and color tests indicated the possible presence of xanthophylls. Comparison of similar chromatograms with authentic standards convinced Villere *et al.* (1944) that no α-carotene was present.

Kemmerer and Fraps (1943) conducted a much more extensive chromatographic investigation of the crude carotene fraction. Ultraviolet absorption spectra of the resolved bands indicated 11.7% of an impurity (perhaps lycopene), an unknown carotene X (5.4%), neo-β-carotene (3.7%), α-carotene (1.4%), and, of course, β-carotene (77.8%). Later data (Kemmerer *et al.*, 1945) on the cooked, raw, and dehydrated product failed to show the α-isomer.

Matlack (1935) reported the isolation of an unidentified phytosterol and a phytosterolin (sterol glycoside). Comparison of Matlack's meager physical and chemical data with the properties of known plant steroids (Table 5) indicates that the glucoside is probably identical with ipuranol, a D-glucoside of Δ^5-stigmastene-3-β-ol ("β-sitosterol") isolated from *Ipomoea purpurea* (Power and Salway, 1913). The phytosterol probably represents the aglycone β-sitosterol.

Several aromatic acids have been reported to occur in sweet potatoes. Rudkin and Nelson (1947) isolated chlorogenic acid and what probably was another closely related ester of caffeic acid. They also obtained evidence, through countercurrent distribution, for the presence of several other dihydric phenols obtained by lead acetate precipitation.

Chlorogenic acid was later isolated by Uritani and Miyano (1955) from sweet potatoes infected with the fungus *Ceratostomella fimbriata*, along with three other caffeic acid esters. One of these was tentatively identified by ultraviolet spectrum and distribution coefficient as isochlorogenic acid, while a similar caffeylquinic acid was given the name "pseudochlorogenic acid"; no "neochlorogenic acid" (Corse, 1953) was detected. It has been suggested that protocatechuic acid (3,4-dihydroxybenzoic acid) may be present (Nishida *et al.*, 1952).

The coumarins umbelliferone (7-hydroxycoumarin) and scopoletin (6-methoxy-7-hydroxycoumarin) also were isolated by column chromatography from the "sound" portion of the diseased tubers (Uritani and Hoshiya, 1953). However, these substances were not present in healthy tubers, as shown by the absence of their strong and characteristic fluorescence. *trans*-Cinnamic acid, too, has been reported to occur only in the diseased vegetable (Kubota, 1958). This appearance of new and rather complex substances after infection undoubtedly has significance in plant disease resistance, and in this respect the sweet potato has received considerable attention. Although the subject may not be strictly applicable to a review of the "normal" constituents of food, it is of such interest and practical significance that it should receive at least a brief discussion.

It has been estimated that diseases destroy 20–40% of the nation's sweet potato crop—in the field, in storage, and in transit to market. Among more than 40 fungus diseases that attack the species, one of the most serious is the "black rot" caused by *Ceratostomella fimbriata*. This problem has been of economic importance for many years, since infected tubers are worse than useless. Animals become sick when fed the spoiled produce, use of the high-carbohydrate mash for fermentative production of alcohol produces an extremely bitter and poisonous by-product, and even the uninfected parts of such diseased tubers are not fit to eat.

Hiura (1947) isolated the bitter principle, an unsaturated ketone, and named it "ipomeamarone," after its botanical source. After several years of investigation, during which a number of erroneous structures were proposed, the substance was shown to be the 3-substituted furan I (Kubota and Matsuura, 1953). Total synthesis (Kubota and Matsuura, 1958) and chemical study of the stereochemistry confirmed the structure and indicated a *cis* relationship of the furan and ketonic side-chain on the tetrahydrofuran nucleus (Kubota, 1958). Additional data (Kubota, 1958) showed

Table 5. Comparison of steroids.

	M.p. (°C)	Acetate m.p. (°C)	Reference
Sweet potato sterolin	285–286	165–165.5	Matlack, 1935
Ipuranol	285–290	164–165	Power and Salway, 1913
Synthetic β-sitosteryl D-glucoside	295–300	166–167	Salway, 1913
Sweet potato sterol	136.5–137	129–129.5	Matlack, 1935
β-Sitosterol	140	127–128	Stecher, 1960

that the natural (+)-ipomeamarone is an optical isomer of (−)-ngaione, previously isolated from *Myoporum laetum* (McDowall, 1925).

The volatile extract of black-rotted sweet potato also contains several other furan derivatives related to ipomeamarone (Fig. 1). Ipomeanine (II) was isolated in a pure state by Kubota *et al.* (1952), and its structure was elucidated by degradation

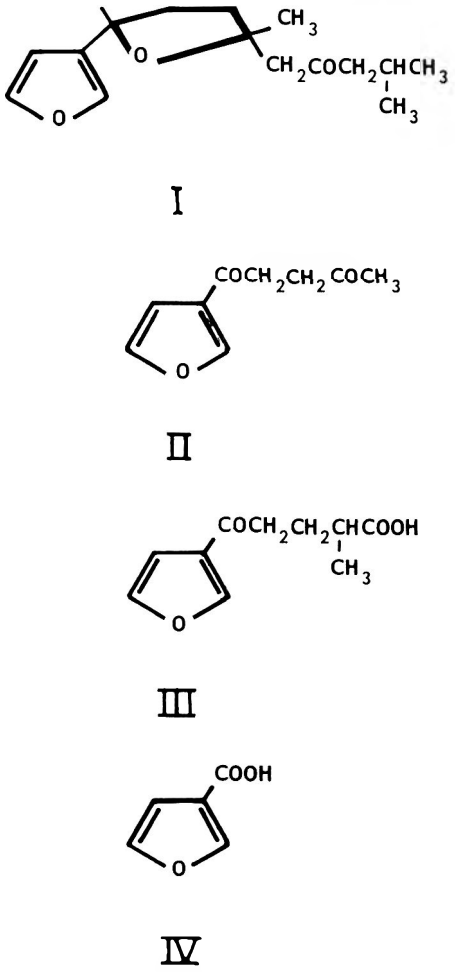

Fig. 1. The furans of diseased sweet potatoes.

and final synthesis (Kubota and Ichikawa, 1954). Batatic acid (III) was likewise clearly identified (Kubota and Naya, 1955) and the structure confirmed by synthesis (Naya, 1956); furan-3-carboxylic acid (IV) was also isolated (Kubota, 1958; Taira and Fukagawa, 1958).

It appears that healthy sweet potato tubers do not contain detectable amounts of furans (Kubota and Sakamoto, unpublished work). It is possible that II, III, and IV may be formed by successive degradation of I in the plant; there is evidence that they are not intermediates in its biosynthesis (Akazawa *et al.*, 1962). Many other probable furanoterpenes have recently been detected in infected tubers (Akazawa, 1960; Akazawa *et al.*, 1962).

DISCUSSION

Despite the extremely limited amount of information available on the chemical constituents of the sweet potato, this instance is illustrative of several important problems which arise in both isolation or detection of plant products and interpretation of the chemical and biochemical data.

First, the investigation of carotenoid content provides an example of the complexity of the isolation problems confronting the experimentalist. What appeared to many workers to be a rather homogeneous pigment proved to be a mixture of closely related components still not adequately resolved or identified. In addition, as an example of the need for caution in interpretation, Ezell and Wilcox (1948) demonstrated that the β-carotene content of the carotenoid fraction of different varieties of sweet potatoes may vary from as much as 85% to as little as 9%, the other components generally having remained unidentified. Varietal differences, so seldom mentioned in the early literature, may frequently be very significant indeed.

Second, the importance of the healthy *versus* the diseased or abnormal state is exemplified by the presence of a number of interesting and unusual constituents—the coumarins and furans—in one case, and their complete absence in the other. These are not necessarily minor differences; the amount of ipomeamarone in an infected tuber may represent more than 2% of the tuber weight (Uritani *et al.*, 1960). Furthermore, disease may not be the only factor involved; the same investigators reported that ipomeamarone was produced when damage was due to mercuric chloride, trichloroacetic acid, or 2,4-dinitrophenol rather than to the fungus!

Actually, such drastic effects of a pathological condition on composition are probably very widespread in the vegetable kingdom. In the recent review on celery constituents (Crosby and Anderson, 1963), the isolation of several potentially toxic furocoumarins was discussed. While the review was in press, Scheel *et al.* (1963) revealed that only

celery infected with pink rot *(Sclerotinia sclerotiorum)* contained detectable amounts of furocoumarins. The normal vegetable was devoid of these compounds. Similar results with other oxygen heterocycles (pisatin and its relatives) have been observed in the garden pea, *Pisum sativum* (Cruickshank, 1963).

Other common plants belonging to the Convolvulaceae are the morning glory, the field bindweed, alkali weed, and the widely used lawn plant *Dichondra*. Although extractives of many of these species have been used as medicinals since before the birth of Christ (Shellard, 1961), very little is known with certainty about their chemistry.

Several anesthetic alkaloids have been isolated from *Convolvulus pseudocantabricus*, the most important of which are the atropine analogs convolvine and convolvamine (Fig. 2) (Orekhov and Konovalova, 1934; Orekhov *et al.*, 1935). The steroid glucoside ipuranol from *Ipomoea purpurea* has been mentioned previously, and an apparently related compound, ipurganol, has been isolated from jalap tubers *(Ipomoea purga)* (Power and Rogerson, 1910).

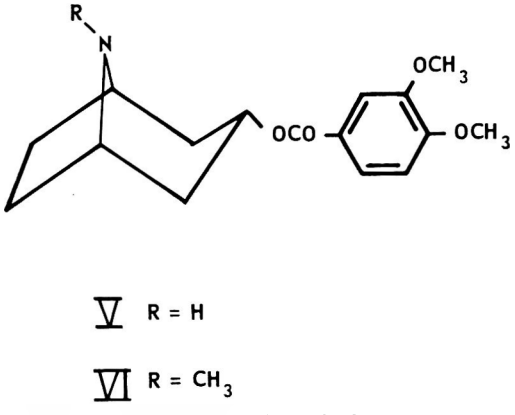

Fig. 2. Alkaloids from *Convolvulus*.

Jalap tubers are also a source of the crude drug known for centuries as "jalap resin" or "jalapin." This resin, as well as that from *Convolvulus scammonia* ("Scammonium"), has a drastic cathartic action. Although popular in early times, the drug is very seldom used today, because of the violence of its effect. The active constituent has never been determined, although a good deal is known about the chemistry of decomposition products of the resin (Power and Rogerson, 1910). The most interesting of these are a series of somewhat unusual fatty acids, including 3,11-dihydroxytetradecanoic acid ("ipurolic acid"), tiglic acid, 11-hydroxyhexadecanoic acid, and 2-methylbutyric acid (Shellard, 1961; Power and Rogerson, 1910; Davies and Adams, 1928).

As noted in the introduction, the sweet potato is one of the world's major food staples. In view of this fact, detailed knowledge about its chemical constitution and the effect of these substances on health and nutrition assumes real importance.

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Malonaldehyde in Aqueous Solution and Its Role as a Measure of Lipid Oxidation in Foods

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SUMMARY

The kinetics of the production of malonaldehyde by acid hydrolysis of its bis-(diethylacetal) is described, and its ionization constants and polymerization in aqueous solution are explored. The frontier electron method from molecular orbital calculations is utilized for prediction of the reactivities of the compound. The addition complex with bisulfite, the reaction with 2,4-dinitrophenylhydrazine, the dimedone derivative, and the ferric chelates were investigated. Finally, the significance of the acidification on the isolation of the compound from foods and its possible role as a measure of lipid oxidation in food systems are discussed.

Malonaldehyde occurs in foods and biological preparations only as a product of lipid oxidation. Moreover, it can be assayed rather simply in aqueous extracts or distillates without the necessity of extracting the lipids. It is the only compound now known that meets both of the above criteria of a good test substance for lipid oxidation in complex foods. Its colored reaction product with 2-thiobarbituric acid (TBA test) has been used very widely to follow the oxidation of tissue lipids. Its reaction with anthrone (Kwon and Watts, 1963a) and its ultraviolet absorption (Kwon and Watts, 1963b) have also been utilized for analysis of the compound in aqueous solutions.

In spite of the unique usefulness of malonaldehyde as a lipid oxidation indicator, the dearth of information on the basic chemistry of this compound and its reactions with other food constituents makes the interpretation of its variations in foods difficult.

Malonaldehyde was obtained by Claisen (1903), but only in aqueous solution through hydrolysis of β-ethoxyacrolein-diethylacetal. Although crystalline malonaldehyde was prepared by Hüttel (1941), it is hygroscopic and volatile, and thus can be kept only a very short time. Since malonaldehyde is not commercially available in the pure form for the above reason, it is customarily obtained as the aqueous solution from acid hydrolysis of its bis-(diacetal)s. However, lack of quantitative data on the rate of hydrolysis and on the stability of its aqueous solutions under various conditions makes even the preparation of standards for further investigative work difficult.

This paper describes the kinetics of acid hydrolysis of malonaldehyde bis-(diethylacetal), and the ionization and polymerization of the product in aqueous solution. The present study further includes a theoretical consideration of the reaction patterns of malonaldehyde from molecular orbital (MO) data and an experimental exploration of several types of reactions that might lead either to improved procedures for isolating it from foods or to a better understanding of its reactivity in food systems.

The expected configurational modifications of malonaldehyde were discussed by Kwon (1963). This methylene interrupted dialdehyde (the diketo form, I) easily undergoes enolization. The enolic tautomer (β-hydroxyacrolein) undergoes molecular rearrangements into its open *s*-cis- (II), *s*-trans- (III), and chelated form (IV). In aqueous solutions, the pH-dependent equilibria further involve the enolate anion (V).

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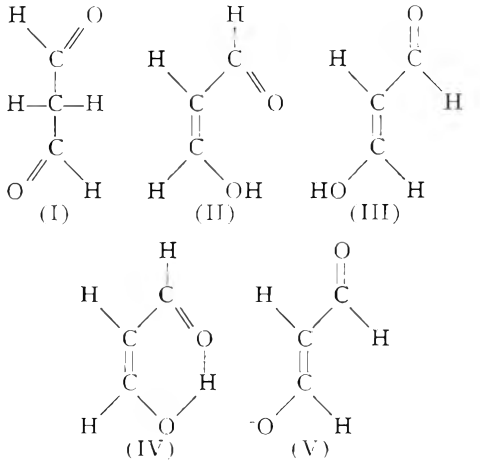


EXPERIMENTAL PROCEDURES, RESULTS, AND DISCUSSION

Hydrolysis of malonaldehyde bis-(diethylacetal). Malonaldehyde bis-(diethylacetal) (Kay-Fries Chemicals, New York 16, N. Y.) was further purified by distilling (Kwon and Watts, 1963b). One liter of the $2.5 \times 10^{-3}M$ solution with 1 ml of $1N$ HCl added as a catalyst was prepared. The solution, in an Erlenmeyer flask, was mounted in a water bath at 48°C. A glass stopper was held in place firmly with stopcock grease to prevent loss of the aldehyde while heating. At 10-min intervals, approximately 15-ml portions of hydrolysate were removed to small Erlenmeyer flasks and cooled in an ice bath. When 10 samples were ready, they were brought to room temperature. To each 10-ml portion was added 0.1 ml of $1N$ NaOH, and the absorbance at 267 mμ, the wavelength of maximum absorption of the enolate anion of malonaldehyde, was recorded against distilled water with a Beckman DU spectrophotometer and a 1-cm cell.

As shown in Fig. 1, the kinetics of the production of malonaldehyde from the hydrolysis are typical of a first-order (Pseudomono-molecular type) reaction. The first-order velocity constant is calculated to be $(k) = 0.0347$ min $^{-1}$ and the time required for the transformation of 50% of product $(t_{1/2}) = 20$ min under these conditions. The acid-catalyzed formation of the enolic tautomer occurs immediately. Mashio and Kimura (1960) pointed out that about 96% of the compound is in the enolic form in aqueous solution.

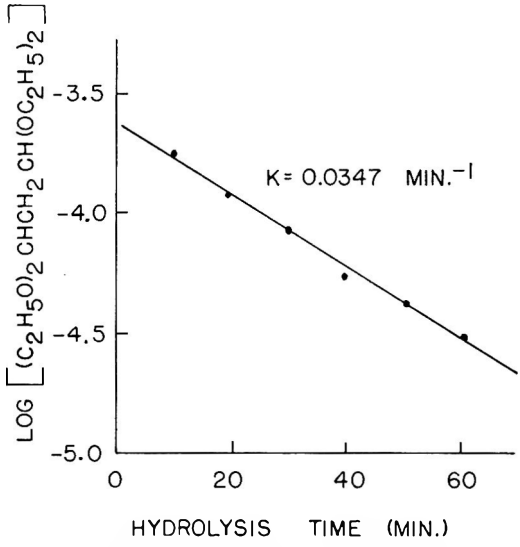


Fig. 1. Plot of a first-order reaction for the production of malonaldehyde from its bis-(diethylacetal).

Yanovskaya and Kucherov (1962) recently investigated the hydrolysis in the presence of an equimolar amount of water. It is interesting to notice that under these conditions the hydrolysis never goes to completion, and in the presence of toluene-*p*-sulfonic acid the hydrolysis leads to products enriched in β-ethoxyacrolein, while in the presence of phosphoric acid, β,β-diethylpropionaldehyde predominates.

Determination of the ionization constant of malonaldehyde. A $1.8 \times 10^{-2}M$ malonaldehyde solution was prepared, according to the procedure described, except for a slightly different acid concentration. Each 100-ml portion of the solution which contained 10 ml of $0.2N$ HCl was titrated with $0.2N$ NaOH, and the pH followed with a Beckman pH meter, Model G. The titration was carried out at several temperatures. The same procedure was followed for blanks containing the same amount of acid. From each set of titration curves, the dissociation curves for malonaldehyde at the temperatures specified were obtained. The dissociation curve is shown in Fig. 2, and the changes in pKa depending on temperature are given in Fig. 3.

An aqueous solution of the compound is known to be acidic (Hüttel, 1941; Mashio and Kimura, 1960). The acidity is rather strong for a pseudo-acid, being almost the

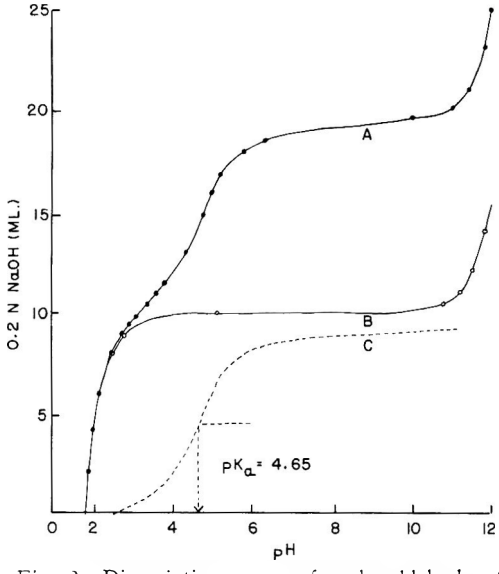


Fig. 2. Dissociation curve of malonaldehyde at 23°C. A) Titration curve of malonaldehyde plus HCl. B) Titration curve of HCl. C) Dissociation curve (difference between A and B).

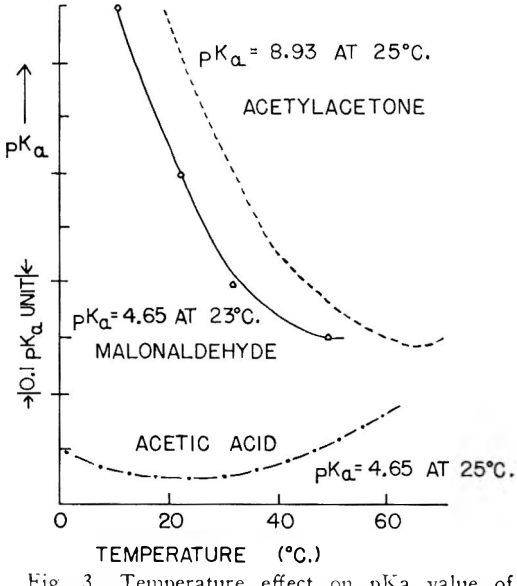
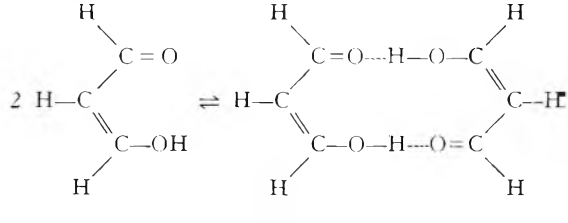


Fig. 3. Temperature effect on pKa value of malonaldehyde. Data for acetylacetone and acetic acid are adopted from Schwarzenbach *et al.* (1940).

same as that of acetic acid at room temperature. Schwarzenbach *et al.* (1940) found that the acidity of acetylacetone increased rapidly with increase in temperature to a maximum, whereas the change for acetic acid was relatively small. From Fig. 3 it may be seen that although the acidity of malonaldehyde is comparable to that of acetic acid at room temperature, the variability of the acidity constant with the temperature is almost parallel to that of the structurally related acetylacetone. According to Schwarzenbach and Lutz (1940) and Schwarzenbach *et al.* (1940), the resonance effect is important in determining the magnitude of the acidity constant. The resonance energy is dependent both on the temperature and on the resonance chain length. Thus, the change in the acidity constant of malonaldehyde with temperature is predictable.

Stability of malonaldehyde. Mashio and Kimura (1960) reported a loss of malonaldehyde from dilute aqueous solution at room temperature. They assumed that polymerization was occurring.

However, dilute solutions (below $2.5 \times 10^{-5}M$) stored in the refrigerator showed no change in malonaldehyde content after 20 days at pH values ranging from 2.3 to 11.3. Controlled hydrolysis of malonaldehyde bis-(diethylacetal) yielded a colorless aqueous solution of malonaldehyde at a concentration up to $3.6 \times 10^{-3}M$, while over-heating resulted in a yellowish color at much lower concentration. When the colorless aqueous solution was allowed to stand at room temperature, a yellowish color gradually formed. Fresh malonaldehyde solutions more concentrated than $6 \times 10^{-3}M$ also were yellowish. The yellowish color was accompanied by an absorption band in the visible range, and its absorptivity increased on heating. The position of the maximum absorption appeared at λ 477 mμ and disappeared upon the addition of acid or base as shown in Fig. 4. The newly formed molecular species could conceivably be a dimer, with intermolecular hydrogen bonds between two open *s*-cis-enol forms of malonaldehyde.



Here a conjugated chelate system (Rasmussen *et al.*, 1949) occurs between the

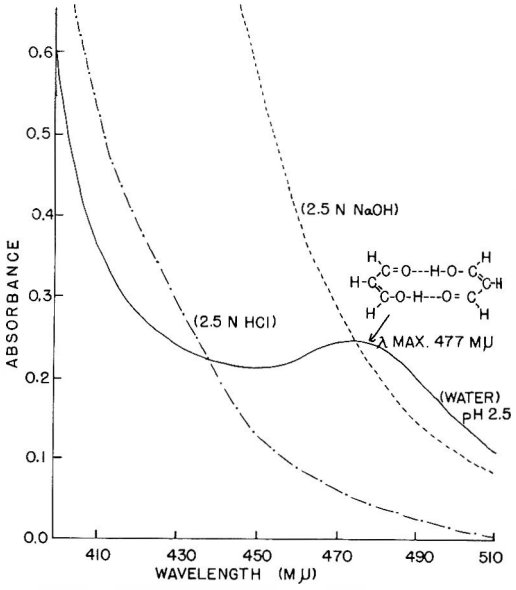


Fig. 4. Absorption spectrum of dimer of malonaldehyde in aqueous solution. Concentration of malonaldehyde: $3 \times 10^{-2}M$. Solvent indicated in parentheses.

molecules. Strong acid or base may destroy the hydrogen bonds in the dimer with loss of the absorption band.

When the aqueous solution ($2 \times 10^{-2}M$) stood at room temperature, a yellow precipitate was formed after about 30 days, which was filtered and dried. The yellowish powder is insoluble in water and in dilute acid, but soluble in concentrated HCl. After further dilution with water (final HCl concentration, $0.24N$), u.v. measurements of the solution showed only about 30% free malonaldehyde. The rest is presumably polymer. Thus, polymerization of malonaldehyde in aqueous solution is enhanced at higher concentrations, higher temperatures, and longer storage periods.

The yellow color of the solutions does not necessarily mean polymerization. Such color may also arise from $n \rightarrow \pi^*$ electronic transitions of the monomer. The absorption band of the $n \rightarrow \pi^*$ transition ($\lambda_{max.} = 350$ mμ) disappears with increasing acidity, while the intensity is enhanced and the maximum moved to 360 mμ with increasing alkalinity (Kwon, 1963). This absorption is enough to give a yellowish color at higher concentrations of malonaldehyde. The yellow colors from the two origins are difficult to distinguish from each other. Thus, freshly prepared malonaldehyde solutions prepared under controlled conditions are yellow only at higher concentrations due to the $n \rightarrow \pi^*$ absorption, while the color from over-heated or aged preparations is due partially to polymerization and partially to $n \rightarrow \pi^*$ absorption.

Prediction of reactivity of malonaldehyde from MO calculations. To a certain extent, the relative reactivity of organic molecules can be predicted from the data of the MO calculation, by the frontier electron method (Fukui *et al.*, 1954). In applying the theory to malonaldehyde, the chelated form is not considered, since this form would be expected to undergo modification before further chemical reactions. From the MO calculation (Kwon, 1963), the orbitals for the molecules considered have the frontier electron densities shown in Table 1.

The electron densities of the highest occupied MO (HOMO) are greatest at the 1 and 5 positions (the terminal oxygen atoms) in the diketo form, at the 3 position (center carbon atom) in the enol form and the anion. Therefore, these are predicted to be the positions most readily attacked by electrophilic reagents. Nucleophilic attack should occur at the 2 and 4 positions in the diketo form and the anion, and at the 4 position in the enol form, from the electron densities of the lowest empty MO (LEMO). For example, the coupling reaction of aromatic diazonium salts with malonaldehyde (Mashio and Kimura, 1962) and the addition reaction of sodium bisulfite with this

Table 1. The frontier electron densities in HOMO and LEMO of malonaldehyde.

Molecule	HOMO					LEMO				
	1 O	2 C	3 C	4 C	5 O	1 O	2 C	3 C	4 C	5 O
diketo form (I)	0.644	0.356	0.000	0.356	0.644	0.356	0.644	0.000	0.644	0.356
enol form (II & III)	0.109	0.005	0.511	0.242	0.133	0.131	0.137	1.166	1.515	0.051
enolate anion (V)	0.143	0.024	0.666	0.024	0.143	0.178	0.322	0.000	0.322	0.178

compound are well explained by the above approach.

Reactions with some inorganic and organic compounds. *Bisulfite addition product.* One of the characteristic properties of aldehydes is their reaction with sodium bisulfite to form addition products. The products are nonvolatile and water-soluble, and the free aldehydes can be recovered by addition of sodium carbonate or HCl. The bound bisulfite can be titrated to give an estimation of total aldehydes (Lea, 1934; Tamsma and Powell, 1959).

Malonaldehyde bisulfite sodium salt was prepared by Saslaw and Waravdekar (1957). The crystals were described as colorless oblong hexagonal plates. These observations were confirmed. As expected from the frontier electron data, the diketo form of the compound appears to be the reactive molecular species in this reaction. Malonaldehyde was regenerated by distillation from the acidified solution.

The possibility of extraction of malonaldehyde from foods with aqueous solutions of sodium bisulfite has been investigated. *N*/12 $NaHSO_3$ (Baker analyzed reagent) solution, 5% HPO_3, and distilled water were compared as solvents. To 10-g portions of refrigerator-stored cooked fish were added 50 ml of each solvent, followed by 5 min of blending in a Virtis "45" homogenizer. Clear extracts were obtained by filtration of the homogenates, and 5 ml of each extract was analyzed for malonaldehyde by the TBA test. Distillates were also prepared from the same sample of fish by the method of Tarladgis *et al.* (1960). The TBA number of the bisulfite extract was about twice that of the HPO_3 and 3 times that of the water extracts. It was slightly higher than that obtained by the distillation method. It is possible that both the bisulfite treatment and the acid distillation may result in cleavage of the cyclic peroxide postulated by Dahle *et al.* (1962) to be a precursor of malonaldehyde. Further work on the purified peroxides is necessary before the results can be interpreted.

Unfortunately, any excess of free sodium bisulfite in the extract reduces the amount of TBA chromogen. Attempts were made to remove the excess sodium bisulfite by using sodium carbonate, sodium bicarbonate, iodine, or hydrogen peroxide in the extracts before the TBA reaction. Hydrogen peroxide (0.003% in final concentration) was reasonably successful for this purpose, but the method was rather cumbersome. In the absence of a simple procedure for analysis of the malonaldehyde bisulfite complex, further work on the extraction of foods with bisulfite was abandoned.

Reaction with 2,4-dinitrophenylhydrazine (DNP). DNP has long been used as a carbonyl reagent. The resulting hydrazones may be separated by paper or column chromatography for further identification of individual carbonyl compounds. The hydrazones usually have specific absorption maxima in the visible region, and this property is often utilized for the quantitative assay of carbonyl compounds. The colors are further changed or intensified by adding rather strong alkali. In particular, dicarbonyl derivatives produce intense colors ranging from red to blue.

An attempt was made to prepare the malonaldehyde-DNP derivative, according to the usual procedures. Malonaldehyde was prepared from its bis-(diethylacetal), and DNP (Eastman) was further purified. The two reagents were reacted in an acidic medium at room temperature, and a red precipitate was immediately formed. The resulting crude powder was dissolved in methanol and chromatographed on Whatman No. 1 filter paper using the upper layer of methanol-heptane (1:2 v/v) (Huelin, 1952). A yellowish-brown spot was observed in the position of $R_f = 0.11$. Rather unexpectedly, no color change was observed after 10% NaOH treatment, and the methanol or chloroform extract of the spot from the chromatogram did not show a definite absorption maximum. Multiple development of the chromatogram yielded another slightly yellow spot of lower R_f value.

Hüttel (1941) has reported that the reaction between malonaldehyde and phenylhydrazine produced 1-phenylpyrazol rather than the bis-hydrazone derivative. The main spot, therefore, is possibly 1-(2,4-dinitrophenyl)- pyrazol, and the second product is unknown. The enolized molecule in an acidic medium is believed to cyclize to

form a new five-membered ring on the benzene ring. From these observations it is unlikely that malonaldehyde contributes to the carbonyl values widely used as measures of lipid oxidation.

Dimedone derivative. The formation of dimedone derivative is another specific reaction of aldehydes (Wild, 1960). Malonaldehyde was found to form characteristic condensation products (yellowish powders) with 5,5-dimethyl-1,3-cyclohexanedione, by simply mixing the dilute aqueous reactants. This reaction should be further investigated as a possible means of extracting malonaldehyde and other aldehydes from foods.

Malonaldehyde-iron chelates. Malonaldehyde reacts with $FeCl_3$ in aqueous solution, producing a characteristic deep red color in an acidic medium, apparently because of complex formation. This color reaction was reported previously by Claisen (1903) and Patton *et al.* (1951).

In order to determine the stoichiometric relation between the reactants, the continuous variation method (Harvey and Manning, 1950; Moore and Anderson, 1945) has been applied. Aqueous $FeCl_3$ (Baker's analyzed reagent grade) and malonaldehyde solutions (both $3.75 \times 10^{-3}M$) were prepared and their pH adjusted to 2.4 by adding dilute HCl. In making absorbance measurements of the complex, the ratio of iron (III) to malonaldehyde was varied with the total molarity of the two kept constant, by mixing various proportions of the two solutions. Color formation was immediate, and the maximum absorption appears at 480 mμ ($\epsilon = 2.1 \times 10^2$).

Absorbance is proportional to the concentration of the complex and passes through a maximum when the ratio of iron (III) to total malonaldehyde in the solution is the same as that in which they react to form the complex. A 1:1 ratio of iron (III) to malonaldehyde was demonstrated (Fig. 5). Recovery of the malonaldehyde by the acid distilling method (Tarladgis *et al.*, 1960), normally used to obtain malonaldehyde from foods, was not affected by iron chelation, even with 10 to 1 ratios of iron to malonaldehyde.

It was noted in earlier work (Foley and Anderson, 1948, 1950) that, as the acidity

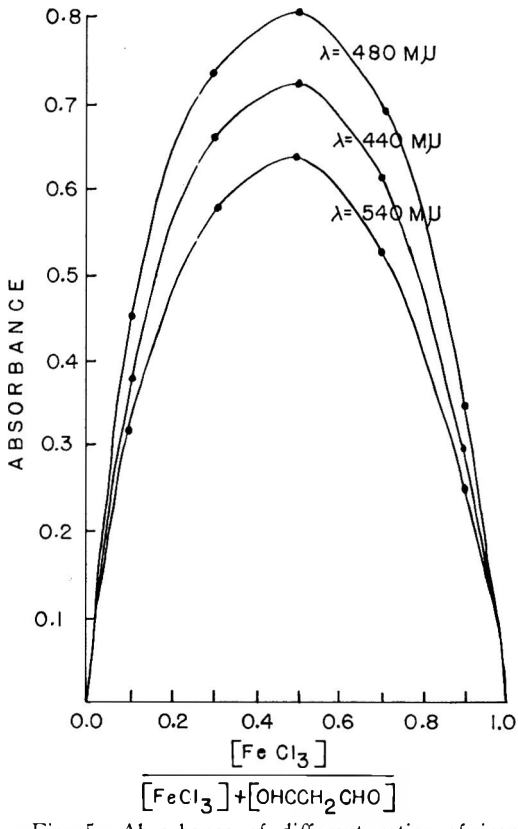


Fig. 5. Absorbance of different ratios of iron (III) to iron (III) + malonaldehyde. Concentrations: $1.875 \times 10^{-3}M$.

decreases, the absorption maximum of the iron chelate solution shifts to shorter wavelengths. For example, in the solution of pH less than 2.4, iron (III) and sulfosalicylic acid react in a 1:1 ratio to form a violet complex, and as the acidity decreases the color of the solution shifts from violet to orange, and eventually to yellow in basic solution (Babko, 1945). This suggests formation of more than one complex, similar to those of the type FeR_2 and FeR_3.

The effect of pH on the color of a malonaldehyde-iron (III) chelate solution ($1.6 \times 10^{-3}M$ final concentration) is shown in Fig. 6. As the acidity decreased upon addition of dilute NaOH, the color faded with progressive precipitation of ferric hydroxide, and a yellowish clear solution was obtained above pH 11. These color changes are attributed to complexes, in which 1:2 and 1:3 ratios of iron (III) to malonaldehyde are maintained. Unfortunately, the exact stoichiometric relation of the reactants

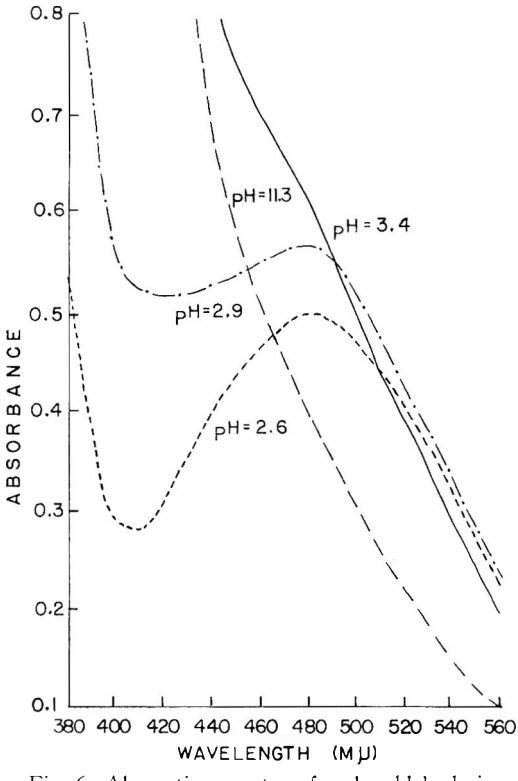


Fig. 6. Absorption spectra of malonaldehyde-iron (III) complexes at different pH. Concentrations: $1.6 \times 10^{-3} M$.

could not be determined at high pH, because of the lack of definite absorption maxima and the instability of the complexes.

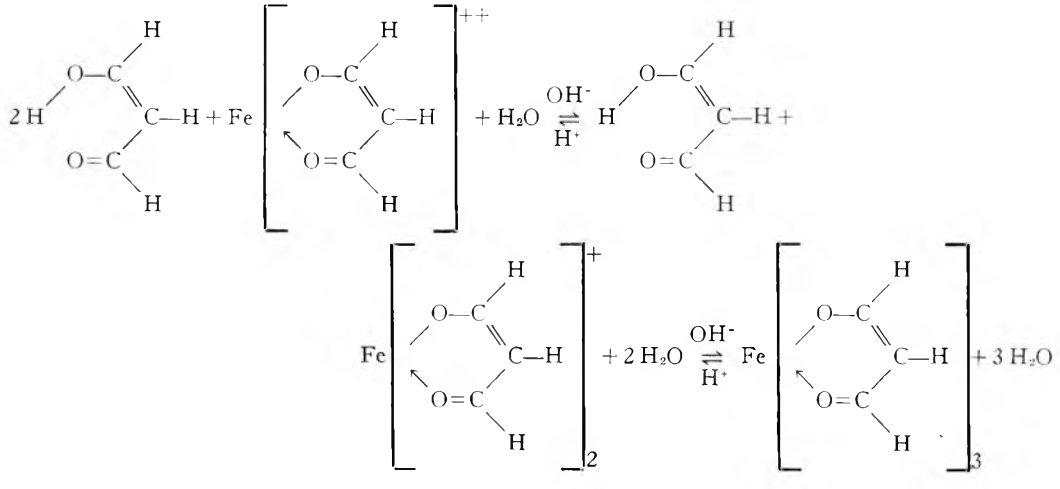
Malonaldehyde forms two links with the central atom, one normal and the other coordinate. With ferric ion, having 6 ligands, the following pH-dependent equilibria may be assumed.

The tri-malonaldehyde-iron (III) complex is expected to have the octahedral configuration, since this corresponds to the greatest separation of ligands and thus should be most stable. It is assumed to be an "outer" complex where $4s4p^34d^3$ orbitals are used, as in the case of the tri-acetylaceton-iron (III) complex (Cartmell and Fowles, 1956).

Djordjevic *et al.* (1962) recently showed that acetylacetone, coordinated to a metal atom, reacts with various reagents. Thus, there is further possibility of reactions between mono-, di-, and tri-malonaldehyde-iron (III) complexes and other chemical components in food systems. Malonaldehyde may also form chelates with other metals in the foods.

Malonaldehyde as a measure of lipid oxidation in foods. Numerous techniques have been used in application of the TBA test to the assay of malonaldehyde in foods. A number of investigators have heated the macerated food directly with an acidified TBA reagent, extracting the pigment with an immiscible solvent (Turner *et al.*, 1954; Yu and Sinnhuber, 1957). Others apply the test to a metaphosphoric or trichloroacetic acid extract of the food (Tappel and Zalkin, 1959) or to a distillate from the acidified food (Sidwell *et al.*, 1955; Tarladgis *et al.*, 1960). All modifications of the method in current use involve an acid treatment of the food.

Where distillation is employed to separate malonaldehyde from other food constituents, maximum volatilization even of free pre-



formed malonaldehyde would not be expected at pH values above 3, since the volatile hydrogen-bonded ring form undergoes progressive ionization with increasing pH from 3 to 6.5 (Kwon, 1963). To test this assumption, the same amounts of malonaldehyde (3.6×10^{-6} moles/100 ml) were distilled at different pH. The results are shown in Fig. 7. The recovery of the

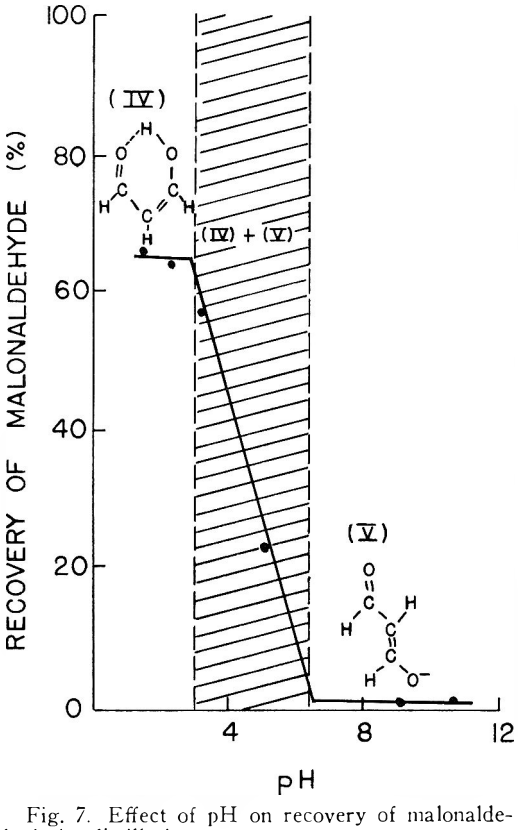


Fig. 7. Effect of pH on recovery of malonaldehyde by distillation.

compound below pH 3 is constant at about 65%, while above pH 6.5 the recovery is negligible. Between pH 3 and 6.5, shown by shaded area in Fig. 7, the recoveries differ dependent upon the proportions of the volatile chelated form (IV) and the nonvolatile enolate anion (V).

Where extraction rather than distillation is used as the initial separation step, water would seem to be an adequate solvent for free preformed malonaldehyde or its metal complexes. The fact that higher yields of malonaldehyde were obtained in fish by an acid extraction or distillation is evidence that the acid treatment either produces more malonaldehyde from a precursor or that preformed malonaldehyde is freed from a secondary combination with some other food constituent by the acid treatment. The extent to which each of these reactions occurs would be expected to depend upon the material being analyzed and upon the condition of storage.

High correlations between malonaldehyde content and rancid odors so far reported in the literature appear to be limited to moist foods, especially animal tissues. In the pH range of such tissues, malonaldehyde produced from lipid oxidation is mainly dissociated into the enolate anion, which is nonvolatile and might conceivably be stabilized against further irreversible reactions by the formation of metal chelates from which malonaldehyde can be recovered by acid and heat. It is interesting to notice that polymerized forms of malonaldehyde might also form metal chelates (Bayer and Fiedler, 1960).

In dehydrated foods, lipid oxidation may be far advanced with little or no accumulation of malonaldehyde. Dehydrated meats, for example, were reported to take up oxygen rapidly and to lose unsaturated fatty acids, with little or no concomitant increase in the TBA reaction (Chipault, 1963). Very little malonaldehyde could be found in highly rancid flour. In such dehydrated foods, the malonaldehyde produced would be expected to be in the volatile chelated form, which would not be held in the food by metal chelation.

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Extraction of Fibrillar and Sarcoplasmic Proteins of Turkey Muscle[a]

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SUMMARY

Protein extraction and fractionation was carried out on samples of breast and leg muscles of turkey males 11–32 weeks old. Nitrogen determinations were made on the total, extractable, coagulable, actomyosin, myosin, and sarcoplasmic protein fractions. These components respectively contained 4.27, 2.57, 1.96, 0.53, 0.09, and 1.36 g nitrogen per 100 g of muscle (wet weight). Nitrogen values were higher for light muscle than for dark muscle.

Muscle samples were taken from 21–36-week-old turkey males both before and after 48 hr at 5–10°C. Results showed a 4.9% decrease in total nitrogen and a 3.4% increase in non-protein nitrogen.

Amounts of protein fractions were compared between birds of three age groups. Birds within the 25–32-week-old group gave highest values for all fractions estimated with the exception of total nitrogen. Turkeys ranging in age from 21–26 weeks gave the highest values for this component. Extractable, coagulable, and nonprotein values increased with increasing age; the 11-week-old turkeys gave the lowest values.

INTRODUCTION

Recognizing that changes in muscle proteins may influence sensory qualities such as tenderness, flavor, and juiciness of meat, procedures have been devised for extraction and fractionation of these proteins (Bate-Smith, 1934, 1937; Hasselbach and Schneider, 1951; Mannan *et al.*, 1951; Robinson, 1952; Helander, 1957; Weinberg and Rose, 1960). To follow changes in muscle proteins involved in quality deterioration of poultry during freezing and storage, Khan (1962) developed a routine method for separation and estimation of these components.

Several changes have been reported in the protein content of chicken meat as influenced by periods of storage. Hepburn (1950) demonstrated that proteolysis occurs in chicken meat with refrigerated storage, and amino acids are produced primarily at the expense of the proteoses and peptones. At cold-room temperature, changes are characterized by increases in total nitrogen of the aqueous extract, plus accumulation of amino acids. Recent work by van den Berg *et al.* (1963) showed that the amount of extractable proteins remains unchanged in breast meat during five weeks of storage at 0°C but increases markedly in leg meat. The effect of growth upon the chemical composition of avian muscle was studied by Dickerson (1960). With increasing age, an increase in the amount of protein nitrogen was observed, and the fibrillar proteins seemed to increase at an earlier age than did the sarcoplasmic proteins.

The objective of this study was to determine the amount of fibrillar and sarcoplasmic proteins in turkey muscle and to observe the effects of storage time and age of turkey on extractability of these proteins.

EXPERIMENTAL

Stock and age of birds used. Keithley White turkey males raised at the Iowa State University Poultry Farm were used in supplying samples for chemical analysis. Age ranges for the turkeys used were as follows: Group I, 11 weeks; Group II, 21–26 weeks; Group III, 25–32 weeks. Six turkeys were selected for analysis from each of groups I and II and five from group III. Comparable nutritional and environmental conditions were maintained in all groups.

Method of exsanguination and sample preparation. All turkeys were slaughtered by cutting the

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jugular vein and carotid arteries, and were bled for 3–5 min. Each bird was scalded for two min at 60°C, and feathers were removed by a commercial automatic circular picker timed for 30 seconds. Following evisceration, pectoralis major and biceps femoris muscles were removed (30 min post-mortem) and were trimmed free of visible fat, tendons, and connective tissues. Two samples were taken at random from each of the two muscles from each turkey, and nitrogen determinations were made in duplicate upon each sample and sample extract. Samples cut from these muscles, representing light and dark meat, respectively, were weighed, wrapped in aluminum foil, and taken to the cold room for extraction. Samples for extraction weighed 2.0–2.2 g, and samples for total nitrogen determinations weighed 20–40 mg. During storage periods, turkey carcasses were left intact to simulate commercial storage conditions.

Muscle analyses. Using the extraction and fractionation scheme of Khan (1962), total, extractable, coagulable, and nonprotein nitrogen were measured in turkey muscle. The effects of age of turkey and refrigerator storage post-mortem were measured with these determinations. Extractable nitrogen was further separated into actomyosin, myosin and sarcoplasmic fractions in samples from turkeys in group III. These steps in analysis, as well as centrifugation, were carried out at 0–5°C.

Nitrogen analyses. All nitrogen determinations were made using a micro-Kjeldahl method (AOAC, 1960). Copper selenite served as the catalyst in digestion, and a methyl red-methylene blue indicator was used in all titrations. Depending upon the protein fraction being estimated, values of percentage nitrogen were multiplied by appropriate dilution factors in order to give results in terms of initial weights of muscle samples. Protein nitrogen was estimated by subtracting nonprotein from total nitrogen.

Storage conditions. Turkeys involved in the storage experiment were stored in plastic bags for 48 hr in a commercial walk-in cooler maintained at 5–10°C.

RESULTS

Protein composition of turkey muscle. The amounts of nitrogen fractions for light and dark muscle are shown in Table 1. Approximately 60% of the total nitrogen was extracted. Of this, 76% was coagulable nitrogen, and 21% was nonprotein nitrogen. Actomyosin, myosin, and sarcoplasmic nitrogen respectively amounted to 27, 4, and 69% of the coagulable nitrogen. With the exception of the actomyosin and myosin fractions, light muscle consistently gave higher nitrogen values than did dark muscle. All muscle differences were statistically significant ($P = .05$) with the exception of the myosin fraction.

Table 1. Mean values for nitrogen fractions of light and dark muscle.

Component	Light muscle		Dark muscle	
Total nitrogen	4.54±.06[a]	(12)[b]	4.00±.06	(12)
Extractable nitrogen	3.02±.16	(24)	2.12±.16	(24)
Coagulable nitrogen	2.28±.12	(24)	1.63±.12	(24)
Nonprotein nitrogen	0.64±.04	(24)	0.44±.04	(24)
Actomyosin nitrogen	0.40±.07	(24)	0.66±.07	(24)
Myosin nitrogen	0.08±.01	(24)	0.10±.01	(24)
Sarcoplasmic nitrogen	1.80±.09	(24)	0.93±.09	(24)

[a] G nitrogen per 100 g of muscle (wet weight) ±standard error of the mean.
[b] Number of determinations.

Effect of storage upon nitrogen fractions. During storage, total nitrogen decreased 4.9%, and nonprotein nitrogen increased 3.4% (Table 2). These differences, though small, were statistically significant ($P = .05$); however, no significant differences existed among the extractable and coagulable nitrogen fractions. Upon storage, the losses in total nitrogen from light and dark muscle were similar. Both extractable and coagulable nitrogen decreased to a slightly greater extent in dark meat than in light, whereas nonprotein nitrogen increased at about the same rate in both muscles.

Table 2. Fresh and stored nitrogen values for light and dark meat.

	Amount[a]			
	Fresh		Stored	
Component	Light	Dark	Light	Dark
Total nitrogen	5.04[b]	4.32	4.76	4.14
Extractable nitrogen	2.91	1.78	2.93	1.70
Coagulable nitrogen	2.20	1.32	2.20	1.22
Nonprotein nitrogen	0.70	0.46	0.73	0.48

[a] G nitrogen per 100 g muscle (wet weight).
[b] Average of six birds representing 12 determinations.

Variation in nitrogen fractions due to age differences. Total nitrogen values were highest in birds from group II, with groups I and III respectively averaging less by 4.8 and 10.2% (Table 3). A similar pattern was observed for nonprotein nitrogen; however, group I data were 13.8% lower than those in group II. In these components, age group differences were found to be statistically significant ($P = .05$). Both extractable and coagulable nitrogen appeared to increase al-

Table 3. Nitrogen values for light and dark muscle in three age groups.

	Amount[a]					
	I		II		III	
	11 wk		21–26 wk		25–32 wk	
Component	Light	Dark	Light	Dark	Light	Dark
Total nitrogen	4.88[b]	4.23	5.03	4.32	4.46	3.96
Extractable nitrogen	2.36	1.58	2.91	1.78	3.06	2.16
Coagulable nitrogen	1.77	1.16	2.20	1.32	2.53	1.68
Nonprotein nitrogen	0.58	0.42	0.70	0.46	0.67	0.45

[a] G nitrogen per 100 g muscle (wet weight).
[b] Average of six birds representing 12 determinations.

most linearly with increasing age. An overall increase of 32.5% was observed in the extractable fraction, while coagulable nitrogen increased 43.7% from group I to group III. In considering the effect of age of bird upon muscle differences, turkeys in group II showed the greatest variation between light and dark muscle. Birds from group III showed similar, but smaller differences, and variation was least in the younger birds of group I.

DISCUSSION

The average total nitrogen value for fresh turkey muscle was 4.27%. This value is somewhat higher than that for chicken meat, for which Dickerson (1960) and Weinberg and Rose (1960) respectively reported values of 3.70 and 3.21%. It appears that a species influence is reflected here. The observation that total nitrogen is generally higher in light muscle than in dark is well supported by the work of Khan (1962), who showed similar results in the chicken. This fact is explained partially by variations in the ratios of nitrogenous constituents to other tissue components such as lipids and water content. It is known, for example, that dark muscle contains a higher percentage of lipids than does light muscle.

Certain morphological and histological differences exist between light and dark meat, such as a higher content of granules and myoglobin in the latter (Walls, 1960). In addition, Frohberg (1963) has shown that, in chicken, dark muscle contains a significantly higher amount of connective tissue than does light muscle. Visual observation in grinding muscle samples supports this latter statement in that dark meat was more resistant to grinding. In general, a more extensive network of connective tissue was observed in the dark muscle. Such difference in amount of connective tissue, plus reported variation in ratios of major muscle constituents, may well account for differences in extractable nitrogen between muscles. Leg muscles are more active physiologically than are breast muscles, and, since the fibrillar proteins are more directly involved in muscular activity than are other components, the amount of this fraction may be expected to increase with activity of the muscle. Walls (1960) stated that the diameter of muscle fibers in muscle increases with activity or exercise. Results of this experiment coincide with such facts in that more fibrillar protein nitrogen could be extracted from dark meat. The relatively small amount of myosin extracted may be explained by the fact that the muscle samples taken for analysis were in the initial stages of rigor, at which time almost all myosin was present in the form of the actomyosin complex.

Almost 22% more sarcoplasmic nitrogen exists in light muscle than in dark. The function of sarcoplasm is directed toward metabolic activities of muscle, since this fraction contains enzymes of the glycolytic system plus various phosphokinases. Thus, no satisfactory explanation can be offered for this finding. Observation of muscle samples, however, shows light meat to have less definitive fibril formation and, perhaps, a more turgid or distended appearance than that of dark muscle. Throughout all experiments, highly significant muscle differences existed in all protein fractions. More nitrogen could be extracted from the breast muscles than from leg muscles of the turkeys.

Two alternatives may be considered in observing the apparent loss of total nitrogen during storage. The decrease could have resulted from either a loss of nitrogen in

the form of free ammonia or from a moisture loss with dissolved nitrogen. It has been generally accepted that ammonia, along with other breakdown products, is formed upon onset and resolution of rigor mortis. Bendall and Davey (1957) have shown that liberated ammonia is attributable solely to the deamination of the adenine nucleotides present in muscle. These workers have further stated that as much as 9 μmoles of ammonia per gram of rabbit psoas muscle may be liberated within 12 hr of storage at 17°C. During storage, increases in the nitrogen content of aqueous extracts of chicken meat have been observed by Hepburn (1950). This increase is due to the accumulation of free amino acids resulting from the degradation of small, simple proteins. This information may also be of value in considering the increase in nonprotein nitrogen during storage.

The data reported here indicate a significantly lower total nitrogen value in turkeys 25–32 weeks old than in younger turkeys. Work by Scott (1956) revealed that percent fat more than doubles during the period from 20 to 26 weeks in Broad Breasted Bronze turkey males. Therefore, such an increase in percent fat could more than compensate for a simultaneous increase in total protein nitrogen. This being the case, the net effect could be an apparent decrease in total nitrogen as observed in this experiment.

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Isolation and Characterization of Low-Density Lipoproteins in Native Egg Yolk Plasma

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SUMMARY

The composition of water-washed yolk granules was different from that of yolk plasma. The lipid content of the granules was about 35%, in contrast to that of plasma (81%). Concentrations of phospholipid, phosphorus-containing protein, and protein nitrogen were not the same for the granules and plasma. A method was developed for the isolation of two ultracentrifugally distinguishable fractions, LPL_1 and LPL_2, from yolk plasma by a differential flotation procedure. The differences in composition of LPL_1 and LPL_2 were small but significant. LPL_1 contained about 89% lipid, whereas LPL_2 was about 86% lipid. LPL_1 and LPL_2 behaved differently when treated by heat and a proteolytic enzyme. A major yolk steroid, isolated by digitonin precipitation, had a melting point and infrared spectrum similar to those for cholesterol.

INTRODUCTION

Egg yolk is a complex mixture consisting of granules that can be detected microscopically and a soluble phase called the plasma. Granules can be sedimented by subjecting undiluted yolk to a centrifugal force of 20,000 × G for 12 hr (Schmidt *et al.*, 1956). Dilution of yolk with water or dilute sodium chloride solution reduces the centrifugation time for sedimentation of the granules into a compact mass (Alderton and Fevold, 1945; Schjeide and Urist, 1959; Burley and Cook, 1961). According to Schmidt *et al.* (1956), the volume of granules in yolk is about 11–13%. On a dry-weight basis, granules represent about 23% of the yolk solids (Burley and Cook, 1961). The work of Burley and Cook (1961) has shown that granules are made up essentially of 70% α- and β-lipovitellins, 16% phosvitin, and 12% low-density lipoprotein. These investigators concluded that low-density lipoproteins are structural constituents of the granules. McCulley *et al.* (1962) showed, by paper electrophoresis, that granule matter is made up of lipovitellins and phosvitin but may be contaminated by γ-livetin if centrifugation is extended unduly. At present, no information is available on the structure of the granules.

Schmidt *et al.* (1956) reported that the plasma of yolk was composed of 77% lipid on a dry-weight basis. According to McCulley *et al.* (1962), plasma with no granule matter contained α-, β-, and γ-livetins as well as a low-density fraction (LDF). Ether extraction of a supernatant with a low-density fraction was found by Fevold and Lausten (1946) to bring about the formation of an insoluble lipoprotein layer between the ether and aqueous phases. The isolated lipoprotein was named lipovitellenin. During ether extraction, so-called free lipid was removed from the supernatant. Several investigators (Weinman, 1956; Evans and Bandemer, 1957; Turner and Cook, 1958) have stressed that all of the yolk lipids are associated with the proteins, and consequently it can be concluded that lipovitellenin (prepared by ether extraction) must be a degradation product. With this fact in mind, it is inadvisable to use ether in a procedure for the isolation of native low-density lipoprotein micelles.

Turner and Cook (1958) presented a flotation technique for the isolation of a low-density lipoprotein fraction (floating fraction) from whole egg yolk. Approximately 80% of the low-density fraction could be removed by ether extraction of its solution. Flotation patterns of the above-mentioned floating fraction suggested that at least two components were present (Martin *et al.*, 1959). According to Sugano and Watanabe (1961), the ultracentrifugal patterns of a low-density lipoprotein fraction,

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prepared by a dilution method, showed two distinct peaks representing components L_1 and L_2. The lipid content of a mixture of L_1 and L_2 in a ratio of 1:2 was 84%. So far, no method has been reported for isolation of the two components in the low-density fraction of the yolk.

The major objective of this investigation was to separate and characterize the low-density lipoproteins in the plasma of the yolk. Some properties of the granules and plasma are presented in this paper since the isolation procedure entails sedimentation of the granules in the yolk and use of the soluble phase as the source of low-density lipoproteins. Digitonin-precipitable steroids in yolk were isolated to prove that cholesterol is a major steroid.

EXPERIMENTAL METHODS

Materials. Eggs were laid by Leghorn hens on a diet that was 14–16% protein and 3.4% corn oil. The eggs were not more than 24 hr old.

Liquid yolk was prepared by the method of Powrie *et al.* (1963).

High-speed centrifugation was used to separate the yolk granules from the plasma (soluble phase). Liquid yolk (25°C) was centrifuged at 20–30°C for about 6 hr at 78,000 × G in a Spinco Model L preparative ultracentrifuge. Refrigeration was turned on for ½ hr at 1-hr intervals. The clear supernatant (plasma) was decanted carefully from the granules. The granules were suspended in about 10 volumes of water and resedimented by centrifuging for 3 hr at 1,000 × G. The granules were washed 3 times.

Preparation of low-density lipoproteins. Fig. 1 is a flow diagram outlining the procedure for the isolation of low-density lipoproteins. A modification of the method outlined by Turner and Cook (1958) was used for the initial separation of low-density lipoproteins from other proteins. One volume of plasma was diluted with 2 volumes of 10% NaCl solution to form a solution. A 17.6-ml portion of this solution was layered with 10 ml of 1.5% NaCl solution in each centrifuge tube, and the mass was centrifuged at 78,000 × G for 5 hr at 5°C. The floating pellicle, P_1, and the cloudy yellow subnatant, T_1, were removed and combined.

The combined fractions ($P_1 + T_1$) were dialyzed 18–24 hr at 5°C against 0.25*M* sodium acetate–acetic acid buffer, pH 4.0, density = 1.013 g/ml (25°C). The resulting solution was centrifuged for 2 hr at 105,000 × G (20°C). Each clear pellicle, P_2, was removed with a spatula from the subnatant, T_2, and dissolved in about 10 volumes of 0.25*M* acetate buffer, pH 4.0. Each subnatant, T_2, was centrifuged for 2 hr at 105,000 × G (20°C), and the resulting floating pellicle, P_3, was combined with pellicles P_2 in solution. The yellow subnatant was removed from each tube with a capillary syringe and was designated T_3.

The combined fractions ($P_2 + P_3$) were dialyzed for 18–24 hr at about 3°C against 0.041*M* acetate buffer, pH 4.0, density = 0.998 g/ml (25°C). The resulting solution was centrifuged for 2 hr at 105,000 × G (20°C). The floating pellicles, P_4, were dissolved in 0.041*M* acetate buffer, pH 4.0, to make a solution with approximately 10% lipoprotein. The solution was dialyzed against 0.041*M* acetate buffer, pH 4.0, and centrifuged for 2 hr at 105,000 × G (20°C). Each resulting pellicle was refloated again in 0.041*M* acetate buffer, pH 4.0. The floating pellicles were dissolved in about 5 volumes of 0.009*M* acetate buffer, pH 4.0, density = 0.997 g/ml (25°C), and the resulting solution was dialyzed for about 18 hr against 0.009*M* acetate buffer, pH 4.0. The solution was centrifuged for 30 min at 105,000 × G (20°C), and a floating scum, insoluble in NaCl solutions, was removed. The low-protein lipoprotein in the subnatants was called LPL_1. The yield of LPL_1 was about 200 mg per 100 g of plasma.

Another lipoprotein was isolated from the subnatant, T_3. The density of subnatant T_3 was adjusted to approximately 1.1 g/ml by adding solid NaCl. The solution was centrifuged at 105,000 × G until the subnatant was colorless (about 3 hr). Each floating pellicle was redissolved in about 5 volumes of 0.25*M* acetate buffer, pH 4.0, and the NaCl content was adjusted to about 10% with solid NaCl. This solution was centrifuged for about 3 hr at 105,000 × G. The pellicles from the centrifuge tubes were dissolved in about 5 volumes of 0.041*M* acetate buffer, pH 4.0. The low-protein lipoprotein in this solution was called LPL_2. The yield of LPL_2 was about 5 g/100 g of plasma.

Isolation of cholesterol. The method of Bergmann (1940) was used to isolate cholesterol from egg yolk.

Analytical methods. Analyses of lipoprotein solutions by ultracentrifugation were performed in a Spinco Model E analytical ultracentrifuge. For each run, a lipoprotein concentration of the solution was about 1%. The flotation rates (—S) were calculated according to the method outlined by DeLalla and Gofman (1954). Paper electrophoresis was conducted in a Spinco Model R Durrum cell with 8 paper strips, S & S No. 2043 A, for each run at 20–25°C. A voltage gradient of 5 volts/cm was maintained. Phosphate buffer, pH 6.5, $\mu = 0.1$, was used in each cell. After electro-

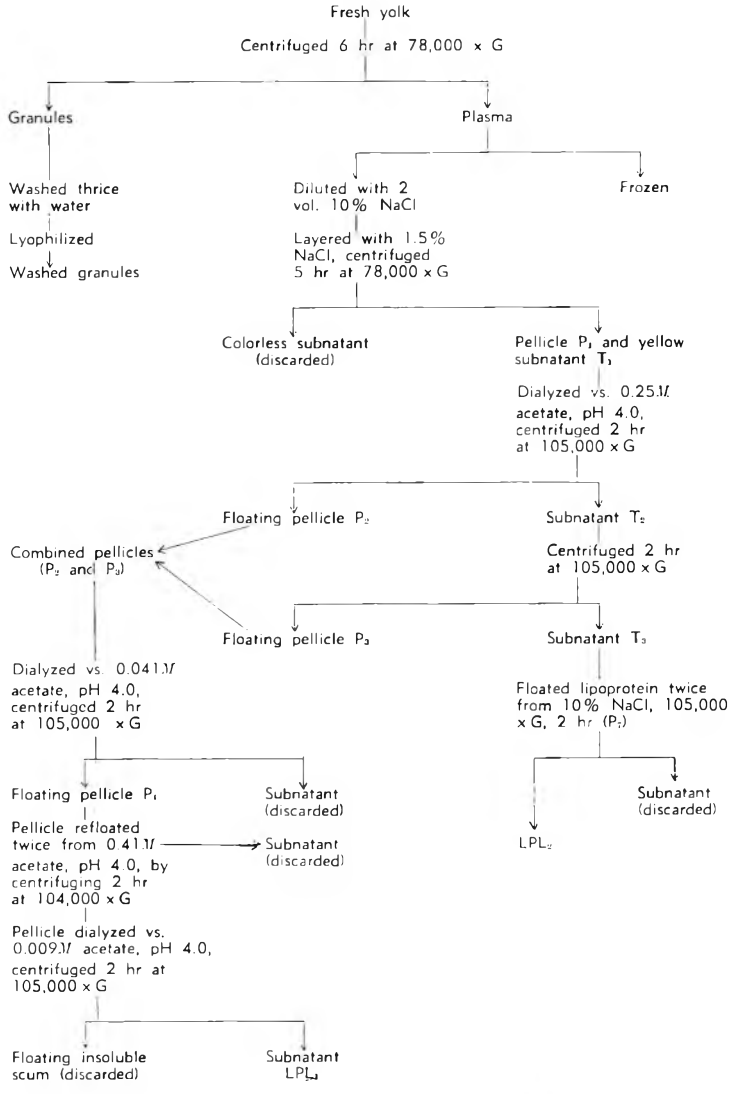

Fig. 1. Flow diagram for the isolation of LPL_1 and LPL_2 from fresh yolk plasma.

phoresis, the paper strips were dried for about 30 min at 120°C and cut longitudinally so that each strip could be stained to detect both protein and lipid. The staining procedures were similar to those outlined by Powrie *et al.* (1963).

Total lipid content of lyophilized lipoprotein samples was estimated by the method of Turner and Cook (1958). Ether-methanol (1:1, v/v) was employed as the extraction solvent.

Nitrogen was measured by the micro-Kjeldahl method described by the AOAC (1955), with $CuSO_4$ as the catalyst. Phosphorus was estimated by the procedure of Chen *et al.* (1956). The method of Sperry and Webb (1950) was used to determine the so-called cholesterol content, and free fatty acids were measured by the procedure of Dyer and Morton (1956). The colorimetric method of Yanagisawa (1955) was used to determine the concentration of divalent cations (as calcium) of samples.

Phospholipids were separated by a thin-layer technique. Aliquots containing 50–80 μg of phospholipids in ether-methanol were spotted on layers of Silica Gel G (Brinkmann Instruments, Long Neck, New York) with a thickness of 0.5 mm. The chromatographs were developed with a chloroform-methanol-water (65:30:4, v/v/v) solvent system (Wagner *et al.*, 1961). The phospholipids were located with sprays of bromthymol blue solution (Jatzkewitz and Mehl, 1960), and 0.5% ninhydrin in 95% ethanol. The appropriate spots were scraped off the glass plate with a razor blade and analyzed for phosphorus.

Used for study of the proteolysis of proteins

by papain were heated and unheated lipoprotein solutions (0.04*M* acetate buffer, pH 4.0). To each 10-ml portion of solution containing 50–200 mg lipoprotein, 3 ml of 0.4*M* Na_3 citrate was added to increase the pH to 6.1. To each substrate sample was added 0.2 ml of enzyme solution (100 mg papain and 5 mg Na_2S in 10 ml of 0.05*M* citrate buffer, pH 6.2). The lipoproteins were digested for 4.5 hr at 37°C.

The TCA-soluble N in each papain digest was determined by adding 13.5 ml of digest to 2.5 ml of 54% trichloroacetic acid solution, filtering the mixture after 1 hr of standing, and analyzing the filtrate for nitrogen content by the micro-Kjeldahl method.

Paper chromatography of nitrogenous constituents in digests was carried out by ascending techniques on Whatman No. 1 paper. Butanol–acetic acid–water (1:1:1, v/v/v) was used as a developing solvent at room temperature. After development, the chromatograms were dried in air at 25°C and sprayed with 0.5% ninhydrin in 95% ethanol.

RESULTS

Granules and plasma. Some ultracentrifugal conditions were studied to develop a simple, rapid method for sedimenting granules from undiluted yolk. With centrifugation at 78,000 × G, effective separation of the granules required only 6 hr at about 25°C but 18 hr at 3°C. Thus, for preparing granules and plasma samples, the centrifuge temperature was maintained between 20 and 30°C.

On a dry-weight basis, the composition of the water-washed granules was markedly different from that of the plasma, as shown in Table 1. In the first place, the lipid content was much higher (81%) for the plasma than for the granules (35%). From the data on the lipid phosphorus content of the granules and plasma it can be concluded that the phospholipid content was significantly higher for granule lipids than for the plasma lipids. The nitrogen content was lower for the nonlipid residue from the plasma than for the granules. Moreover, the nitrogen contents of the nonlipid residues from both yolk fractions were lower than that normally expected in proteins. The low nitrogen value for the nonlipid residue of the granules and plasma may be accounted for by the presence of large amounts of mineral matter and carbohydrate (Abraham *et al.*, 1960; Ito and Fujii, 1962). A 10-fold difference existed between the plasma and granules in the phosphorus content of nonlipid residues. The high phosphorus content of the granule nonlipid residue is undoubtedly due to the presence of a phosphoprotein, phosvitin (Burley and Cook, 1961). With the 0.5% divalent cation concentration (as calcium) in the granules (Table 1), a large number of intermolecular bridges between phosphate groups would be expected in phosvitin.

Table 1. Analysis of water-washed granules and plasma.

Constituents	Granules[a] Percent[b]	Granules[a] s[c]	Plasma[a] Percent[b]	Plasma[a] s[c]
Lipid	34	1	81	2
Lipid phosphorus[d]	1.5	0.1	1.2	0.1
Phospholipid (P × 25)[d]	37	3	29	3
Nonlipid residue	64	1	18	1
Residue phosphorus[e]	1.9	0.1	0.2	0.1
Residue nitrogen[e]	14.8	0.2	12.8	0.4
Ash	5.9	0.1	2.2	0.1
Divalent cations, as calcium	0.5	0.1	0.16	0.04

[a] Moisture-free.
[b] Mean of at least three determinations, and in most cases more than five determinations.
[c] Standard deviation.
[d] Percent of lipid.
[e] Percent of nonlipid residue.

The composition of granules in relation to the whole yolk solids is presented in Table 2. The values of this study are similar, but not identical, to those of Burley and Cook (1961).

Physical characteristics and composition of low-density lipoproteins. The flotation patterns for purified LPL_1 and LPL_2 are shown in Figs. 2 and 3. The broad peak of LPL_1 indicates that this fraction contained a large number of closely-spaced migrating boundaries. This peak broadening is typical of low-density lipoproteins. LPL_2 has a sharper and more symmetrical peak. The flotation rates (—S) for LPL_1 and LPL_2 were respectively 76 and 52.

Paper electrophoresis was used to assess the homogeneity and migration of the protein fractions. As shown in Fig. 4, protein bands containing no lipid were absent in electrophoretograms for purified LPL_1 and LPL_2 fractions. Tailing of all electrophoretograms indicates that lipoproteins were strongly adsorbed to the filter paper. It

Table 2. Composition of yolk granules in relation to whole yolk.

	This study (%)	Burley and Cook (1961) (%)
Yolk solids	19	23
Yolk phosphorus	35	41
Yolk protein phosphorus	89	91
Yolk divalent cations as calcium	59	68
Yolk lipids	9	10

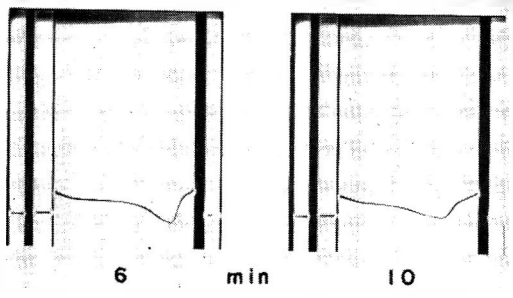

Fig. 2. Ultracentrifugal flotation pattern of LPL_1. 1.75M NaCl–0.05M citrate buffer, pH 6.2, density = 1.070 g/ml (25°C), 33,000 rpm at 21°C.

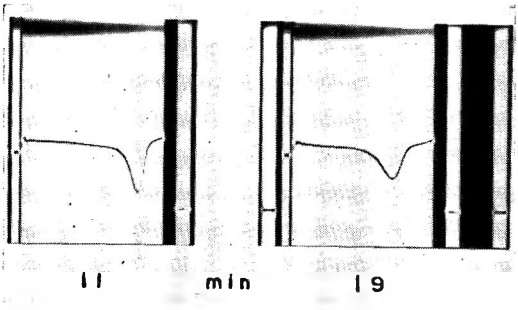

Fig. 3. Ultracentrifugal flotation pattern of LPL_2. 1.75M NaCl–0.05M acetate buffer, pH 4.0, density = 1.068 g/ml (25°C), 42,000 rpm at 21°C.

is of interest to note that the migration of LPL_2 is dependent on the amount of lipoprotein applied to the paper. Studies indicated that the migration of LPL_1 was much lower than that of LPL_2 under comparable conditions. Attempts failed to resolve a mixture of LPL_1 and LPL_2 by paper electrophoresis.

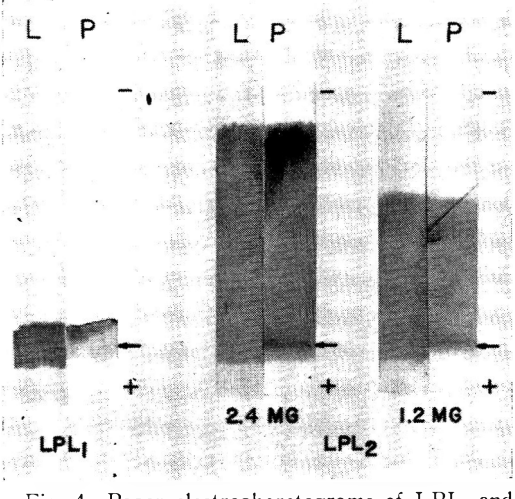

Fig. 4. Paper electrophoretograms of LPL_1 and LPL_2. Electrophoretic conditions: phosphate buffer, pH 6.5, ionic strength = 0.1, voltage gradient = 5 v/cm for 24 hr. Amount of sample applied to each strip: 0.3 mg of LPL_1, 1.2 and 2.4 mg of LPL_2. P = protein stain, L = lipid stain.

Small differences were found in the composition of LPL_1 and LPL_2, as shown in Table 3. LPL_1 contained about 3% more total lipid and about 3% less phospholipid (on a lipid basis) than LPL_2. The molar ratio of lipid N to lipid P in the complexes would be expected to be very close to 1.00 when the concentration of sphingomyelin is low. The ratio of 1.14 for LPL_2 is larger than expected. The high value may have been caused by the extraction of free amino acids adsorbed to the phospholipids. Amounts of phosphorus in the nonlipid residues of LPL_1 and LPL_2 were significant. Consequently, it may be assumed that some esterified phosphate groups were present in the protein moieties. Both LPL_1 and LPL_2 gave a positive Molisch test for carbohydrate.

The concentrations of phospholipids in LPL_1, LPL_2, and granules are presented in Table 4. The values for the 3 fractions are similar and show that phosphatidyl choline is the predominant phospholipid. The PC-PE ratio ranged from 6.5 to 5.7. According to the data of several investigators (Rhodes and Lea, 1957; Privett *et al.*, 1962), the ratio of phosphatidyl choline to phosphatidyl ethanolamine for whole yolk was about 5. On the other hand, Evans and Bandemer (1961) reported that in yolk the ratio was 44.7% phosphatidyl choline and 55.3% phosphatidyl ethanolamine. Two tailing phospholipid spots A and B on the thin-layer chromatograph were not identified. Privett *et al.* (1962) presumed that these spots are sphingomyelin and lysophospholipids.

Cholesterol in egg yolk. Up to the present, no conclusive proof has been presented for the pres-

Table 3. The composition of lipoprotein fractions isolated from native yolk plasma.

Constituents	LPL_1 Mean	LPL_1 s[a]	LPL_2 Mean	LPL_2 s[a]
Total lipid, %	89	2	86	2
Lipid phosphorus,[b] %	1.00	0.03	1.14	0.03
Phospholipid (25 × P),[b] %	25.0	0.1	28.2	0.1
Lipid molar N/P ratio	1.04	0.05	1.14	0.1
Cholesterol,[b] %	3.3	0.3	3.4	0.4
Free fatty acid[b] (as stearic), %	5	1	4.5	0.5
Nonlipid residue, %	11	1	14	1
Residue nitrogen,[c] %	14.8	0.2	13.7	0.5
Residue phosphorus,[c] %	0.15	0.05	0.16	0.05

[a] Standard deviation.
[b] Percent of lipid.
[c] Percent of residue.

Table 4. Separation of the phospholipids in egg yolk fractions by thin-layer chromatography.

	LPL_1		LPL_2		Granules	
Phospholipids	Mean	s[a]	Mean	s[a]	Mean	s[a]
Phosphatidyl ethanolamine,[b] %	13	1	14	1	15	1
Phosphatidyl choline,[b] %	84	1	83	1	82	2
Spot B,[b] %	1.9	0.5	1.5	0.2	1.9	.....
Spot A,[b] %	1.8	0.5	1.4	0.4	2.4	.....
PC/PE ratio[c]	6.5	0.5	6.0	0.5	5.7	0.5

[a] Standard deviation.
[b] Percent of total lipid phosphorus.
[c] Ratio of phosphatidyl choline to phosphatidyl ethanolamine.

ence of cholesterol in yolk. Thus, it is not known whether the steroid(s) in LPL_1 and LPL_2 were cholesterol since the colorimetric method used for the determination of steroids (expressed as cholesterol) was not specific for pure cholesterol. Thus, a study was initiated to isolate the steroid fraction from egg yolk and to show that pure cholesterol was a major steroid in yolk.

Steroids were precipitated by digitonin so that they could be separated from the other yolk lipids. About 800 mg of washed digitonide was obtained when 700 mg of digitonin was added to a yolk lipid extract containing about 3 g of steroid, determined as cholesterol by the colorimetric method of Sperry and Webb (1950). Approximately 40 mg of a waxy substance was separated from the digitonide by the splitting procedure of Bergmann (1940). The recrystallized substance had a melting point of 147°C. According to Feiser and Feiser (1949), the melting point of cholesterol is 149°C. Comparison of the infrared spectra of the isolated yolk steroid and pure cholesterol (Fig. 5) indicated that both of these compounds had the same structure.

Some properties of LPL_1 and LPL_2 fractions. The major plasma fraction, LPL_2, was very soluble in acetate buffers of low ionic strength, pH 4.0. LPL_2 solutions with solids in excess of 15% were not turbid initially and remained clear during a 15-day storage period at 0–5°C. Clear solutions of 5% LPL_1 were prepared with a 0.009*M* acetate buffer, pH 4.0. When a fairly high concentration of salt was present in an acetate buffer, pH 4.0, LPL_2 and LPL_1 micelles tended to aggregate. For example, a floating scum was observed after 1% LPL_2 in 1.75*M* NaCl–0.01*M* acetate buffer, pH 4.0, stood for 48 hr at 0–5°C. Of particular interest, a floating scum formed within a few minutes at the beginning of dialysis of a 1% LPL_1 solution against a 1.75*M* NaCl–0.01*M* acetate buffer, pH 4.0. However, by using a 1.75*M* NaCl–0.05*M* citrate buffer with a pH of 6.2, a

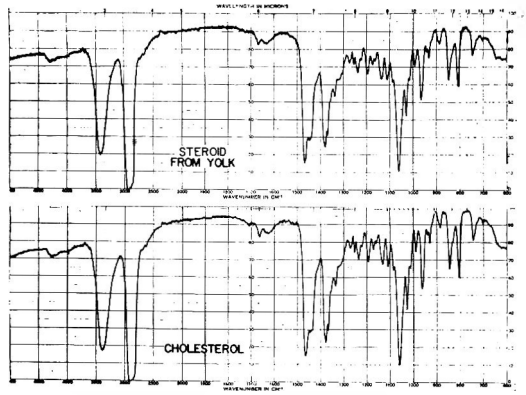

Fig. 5. Infrared spectra of yolk steroid and cholesterol. Samples prepared by KBr pellet method.

1% LPL_1 solution remained stable for 24 hr. For ultracentrifugal flotation experiments, therefore, the LPL_1 was solubilized in citrate buffer, pH 6.2.

Generally, heat energy will rupture hydrogen bonds and other weaker bonds in a system. Thus the disruption of the LPL_1 and LPL_2 micelles in heated aqueous solutions would be expected. When lipoproteins LPL_1 and LPL_2 were heated in a buffer of low ionic strength, pH 4, no appreciable increase in optical density was observed. However, as the ionic strength of the solution increased, the turbidity of the heated lipoprotein solutions increased (Fig. 6). LPL_2, with a higher proportion of nitrogenous constituents, appeared to be more heat-sensitive than LPL_1 at the same ionic strengths. Additional information regarding the effect of heat on the LPL_2 solution was gained by paper electrophoresis. As shown in Fig. 7, the electrophoretic mobility of LPL_2 was reduced when this lipoprotein was heated in a buffer of low ionic strength, pH 4.0. When the lipoprotein solution with 3% NaCl in the buffer was heated, the solution became opaque, and the electrophoretic mobility of LPL_2 was reduced to zero.

It is reasonable to assume that proteolysis of the lipoprotein micelle could occur if the polypeptides were on the surface and if the polypeptide conformation was suitable for proteolysis. Table 5 shows that native LPL_2, but not native LPL_1, was decomposed by papain. However, when the lipoprotein solutions were heated in a buffer of low ionic strength, large amounts of TCA-soluble N were released when either LPL_1 or LPL_2 was treated with papain. It is of interest to note the relatively high concentration of TCA-soluble N for native LPL_1 with no enzyme treatment.

One-dimensional paper chromatography of the TCA-soluble N filtrates of papain-digested lipoproteins showed no new ninhydrin-positive compounds compared to the chromatograms of un-

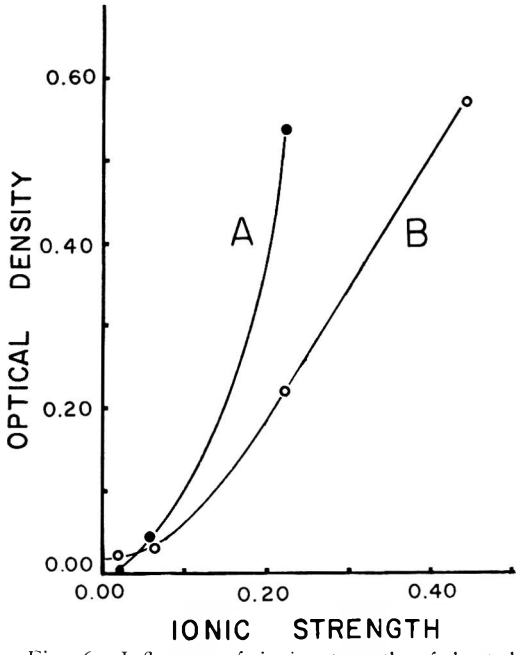

Fig. 6. Influence of ionic strength of heated LPL_1 (curve B) and LPL_2 (curve A) solutions on the optical density at 700 mμ. Final lipoprotein solids content for each solution was 0.12%.

treated lipoprotein solutions. The TCA-soluble N compounds of the untreated and papain-treated lipoproteins formed a diffuse ninhydrin-positive area between R_f values of 0.2 and 0.8. These results indicate that many free amino acids and/or small peptides were present in both the native and enzyme-digested lipoprotein micelles.

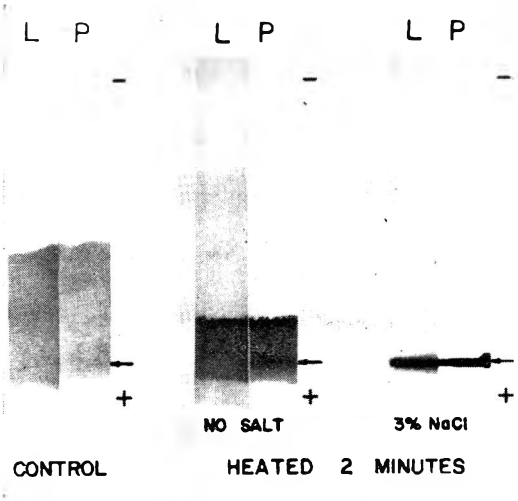

Fig. 7. Paper electrophoretograms of LPL_2, unheated and heated in 0.04 *M* acetate buffer, pH 4.0. Heat treatment at 100°C. Electrophoretic conditions: phosphate buffer, pH 6.5, ionic strength = 0.1, voltage gradient = 5 v/cm for 18 hr. Amount of sample applied to each strip: 2 mg LPL_2. P = protein stain, L = lipid stain.

DISCUSSION

Knowledge on the properties of granules and soluble phase should be valuable for explaining the alteration of physical properties of egg yolk during processing treatments such as pasteurization, freezing, and dehydration. Proteins in the soluble phase are presumably more susceptible to such processing than granule proteins, which are aggregated and exist in large insoluble particles. According to some reports, the lipoproteins in the soluble phase are altered by the freezing and thawing of yolk (Fevold and Lausten, 1946; Powrie *et al.*, 1963).

Table 5. Effect of papain treatment on the concentration of TCA-soluble N of heated and unheated solutions of LPL_1 and LPL_2.

Lipoprotein fraction	Mg TCA-soluble N per 100 mg total protein N	
	With heated enzyme [a]	With active enzyme [a]
LPL_1	18	18
LPL_1, heated [b]	20	73
LPL_2	2	36
LPL_2, heated [b]	5	55

[a] Lipoprotein solution incubated 4.5 hr at 37°C.
[b] Lipoproteins in 0.04 *M* acetate buffer, pH 4.0, were heated 5 min at 100°C.

The composition of yolk granules is dependent on the method of preparation, particularly the washing procedure (Burley and Cook, 1961). The purpose of the granule washing is to remove the proteins of the soluble phase (plasma). In this study, water, rather than a dilute sodium chloride solution, was used as the washing medium, to ensure a minimum loss of granule proteins. Sodium chloride in the wash water would be expected to solubilize some surface proteins. The lipid contents of granules isolated by Schmidt *et al.* (1956) and Burley and Cook (1961) were lower than the lipid content of granules prepared in this study. The differences in the lipid concentration of the granules may be due to the variation in the content of the low-density lipoprotein, which is presumably an integral part of the granules.

The soluble phase, with a moisture content of about 49% (from this study), contains solubilized lipoproteins and nonlipid

proteins. According to McCully *et al.* (1962), plasma consists of α, β, and γ-livetins as well as a low-density lipoprotein fraction. The high lipid content of the plasma solids (Table 1) is indicative of the presence of lipoproteins with a high-lipid: low-protein ratio. The lipid concentration of crude low-density lipoprotein isolated from yolk has been estimated by Turner and Cook (1958) to be about 90%, and by Sugano and Watanabe (1961) to be 84%. Since the crude lipoproteins were fractionated from whole yolk by the above-mentioned investigators, some of the low-density lipoprotein of the granules may have been included in the isolated fractions along with the plasma lipoproteins. Low-density lipoproteins, in general, have been considered as aggregates or micelles composed of a lipid core with a protein film on the surface (Cook and Martin, 1962).

Heterogeneity of the low-density protein fraction of egg yolk has been suggested by Martin *et al.* (1959), Sugano and Watanabe (1961), and McCully *et al.* (1962). In our study, the flotation pattern of a crude low-density lipoprotein fraction showed 2 broad peaks, indicating that 2 distinct fractions, with different flotation characteristics, were present in the plasma.

Chemical analyses of LPL_1 and LPL_2 indicate that surface-active agents, including free fatty acids as well as phospholipids, made up about 30% of each lipid residue (Table 3). Sugano and Watanabe (1961) reported a phospholipid content (on a lipid basis) of 25% for a crude low-density lipoprotein. Undoubtedly, these surface-active agents, along with proteins, stabilize the structure of the micelles. Notably, the presence of micelles without protein has not been established (Martin *et al.*, 1959). Evidently both LPL_1 and LPL_2 contain phosphoproteins since each nonlipid residue contained about 0.15% P (Table 3). In the crude low-density lipoprotein prepared by Sugano and Watanabe (1961), about 0.3% P was determined. Since lipid-free protein (vitellenin) of low-density lipoprotein in yolk possesses at least 2 polypeptide chains (Martin *et al.*, 1959), LPL_1 and LPL_2 may possess different proteins.

Knowledge is meager on the structural characteristics of lipoproteins in general. The lipoprotein micelles are considered to be spherical. Sugano and Watanabe (1961) concluded from experimental data that particles L_1 and L_2 of a low-density yolk lipoprotein were spherical and had respective hydrated diameters of 310 and 250Å. They estimated the respective molecular weights of L_1 and L_2 to be 9×10^6 and 4.8×10^6. With such a low concentration of protein in LPL_1 and LPL_2, the surfaces of the micelles are probably not completely covered by proteins, but have some polar groups of phospholipids (Cook and Martin, 1962). With protein on the surfaces of micelles, proteolysis by enzymes would be expected under controlled conditions. LPL_2 was susceptible to enzymatic attack by papain during a 4.5-hr digestion period. On the other hand, no TCA-soluble N was released from LPL_1 with papain treatment. Since LPL_1 contained less protein than LPL_2, protein-phospholipid interactions may be predominant and render the polypeptides resistant to proteolysis. LPL_2, with more protein, may consist of micelles with large surface protein-protein aggregates that are susceptible to proteolysis by papain. Although proteins or polypeptides have been considered as the nitrogenous constituents on the micelle surfaces, significant amounts of peptides may be present on LPL_2 and, particularly, LPL_1. Such a concept is supported by data on TCA-soluble N in lipoprotein samples with no enzyme treatment (Table 5).

According to Gurd (1950), physical and chemical treatments of lipoproteins cause the disruption and aggregation of micelles, and, as a consequence, solutions become turbid from the formation of large particles. When lipoprotein LPL_2 solutions (pH 4.0) with low ionic strengths were heated, the optical densities of the solutions were not altered. Although no large particles formed, evidence for the disorganization of the micelles by heat treatment was obtained by examining the electrophoretic mobility (Fig. 7) and susceptibility of the heated lipoprotein to papain (Table 5). Undoubtedly, surface protein was cleaved from the phospholipid layer of the micelles and thus,

proteolysis was enhanced in the cleavage areas. When the ionic strength of native LPL_1 and LPL_2 solutions was increased by the addition of NaCl, the aggregation of micelles during the heat treatment was enhanced and the solutions became turbid. Presumably the addition of small amounts of NaCl decreased the positive charges on the micelles and allowed aggregation to proceed.

ACKNOWLEDGMENT

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Chromatographic Fractionation of Sarcoplasmic Proteins of Beef Skeletal Muscle on Ion-Exchange Cellulose[a, b]

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SUMMARY

Sarcoplasmic proteins from beef skeletal muscle were fractionated by chromatography on ion-exchange cellulose (cellulose phosphate and diethylaminoethyl cellulose) at pH 5 and 9.3. From effluent diagrams of sarcoplasma from muscle immediately after slaughter, at least 14 fractions were recognized, and some of them were identified with known proteins by assays for enzymic activities. The numbers and levels of eluted peaks in the effluent diagram were found to decrease with storage (aging) of muscle and freeze-drying of sarcoplasma; these decreases are perhaps due to denaturation. Proteins in solutions appear to be more completely separated by chromatography when different combinations of ion-exchange cellulose are used.

INTRODUCTION

Many of the individual proteins of skeletal muscle have been studied in great detail, but the relative importance of these proteins in relation to the qualities of meat is still obscure. The conditions for the study of individual proteins from muscle are quite different from the conditions that hold for these same proteins in meat. Furthermore, in order to understand the actions and interactions of proteins in meat as it is aged, processed, etc., it is of importance to know the relative amounts of individual proteins or groups of proteins in a particular muscle or sample of meat. Chromatography of muscle proteins offers a possibility that they could be separated quantitatively. However, methods would be needed that are not excessively time-consuming, since meaningful information would require examination of a large number of muscles under various conditions. We are reporting here results of investigations of a single-pass chromatographic technique that offers some promise for meat investigations. It will be seen that the procedure is capable of picking up differences in muscle and changes in muscle proteins with aging, although not all enzymes are separated completely in one pass. Nevertheless, the technique shows a fair measure of reproducibility, and 90–95% of the proteins placed on the column are recovered in the various fractions.

As a starting point for this work we elected to work first on the sarcoplasmic proteins rather than the myofibrillar proteins, because of the ease of prepairng this major fraction of muscle proteins. This was done even though most of the studies carried out so far on post-mortem changes of meat during aging have been concerned primarily with the proteins of the contractile system, the myofibrillar proteins. To be sure, the sarcoplasmic proteins do not play a direct role in contraction, but they reflect some aspects of the post-mortem changes occurring in meat, and therefore have some relation to the specific properties of meats. Relatively little systematic experimentation has been made to develop methods for fractionation and identification of the individual components from sarcoplasma. The anion-exchange cellulose, diethylaminoethyl cellulose (DEAE-cellulose), has proved particularly useful for fractionation of the components of proteins of complex biological materials, such as egg white proteins (Mandeles, 1960) and milk proteins (Yaguchi *et al.*, 1961). With egg white proteins the patterns obtained by chromatography on DEAE-cellulose showed more detail than

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the patterns by conventional electrophoresis (Mandeles, 1960).

The present paper presents results on the fractionation of sarcoplasmic proteins of skeletal muscle on ion-exchange cellulose. This procedure is applied to the study of changes in the sarcoplasma resulting from freeze-drying, salt infusion, and storage (aging) of muscle. Certain proteins within some of the fractions were identified.

EXPERIMENTAL

Preparation of muscle extract. Animals were slaughtered by the usual procedure in the Meats Laboratory of the Department of Animal Science. Within about 60 min post-mortem, the desired muscle was excised, placed in polyethylene film, and cooled in crushed ice for transport to the laboratory. One hundred grams of the chilled muscle, after removal of external fat and connective tissue, was homogenized for 2 min in a Waring blender with an equal weight of cold double-distilled water, and the homogenate was then centrifuged for 20 min at 10,000 × G. The supernatant, under continuous slow agitation with a magnetic stirrer, was dialyzed overnight against cold water. After dialysis, the supernatant was clarified by centrifugation for 20 min at 10,000 × G.

Chromatography. Cellulose phosphate (1.12 meq/g) and DEAE-cellulose (Type 20, 0.70 meq/g) were both purchased from Brown Company, Berlin, New Hampshire. Before use, cellulose phosphate was washed successively on a Buchner funnel with 1*N* NaOH, water, and the starting buffer solution for the elution. As the usual starting-elution solution, 0.1*M* phosphate buffer solution at pH 5 was used with good fractionation. Prewashing of DEAF-cellulose was carried out as reported by Mandeles (1960).

Twenty-five or 30 ml of the dialyzed muscle extract, mixed with an equal volume of the starting elution solution, was chromatographed on cellulose phosphate (4 × 25-cm column) by stepwise elution (flow rate, about 1 ml per min) with buffers ranging from pH 5 to 11 (Rhodes *et al.*, 1958). One freeze-dried preparation fractionated from muscle extract was rechromatographed on DEAE-cellulose (2 × 40-cm column). The elution schedule was carried out as reported by Hartshorne and Perry (1962). The effluent was collected by a fraction collector with drop counting system (volume, 10 or 20 ml), and examined at 280 mμ in a Zeiss spectrophotometer. All experiments were carried out at 1–3°C.

Total protein recovered was calculated to be more than 90%, including a fraction eluted with 0.5*N* NaOH, as determined from measurement of optical density. Protein content was determined by semimicro-Kjeldahl method, and pH values were measured by Beckman Zeromatic pH meter. For enzyme assays, fractionated portions (Fraction I–X, bottom chromatogram of Fig. 1) were dialyzed overnight against cold water, and after dialysis were centrifuged, freeze-dried in a laboratory freeze-drying apparatus, and kept in a deep freezer.

When duplicate extractions and chromatograms of the same muscle were made, reproducibility was good in view of the complexity of procedure and the material being studied. Most of these repeated experiments are not shown. However, the bottom chromatograms in Figs. 9, 10, 11 indicate the reproducibility of the whole procedure by comparing the water extract of salt-infused semimembranosus with salt extract of uninfused paired muscle from the same animal (Figs. 9, 10) and water and salt extracts from the same muscle (Figs. 10, 11). Increasing the amount of pH 6 buffer did not improve separation markedly, as shown in Figs. 9, 10, 11, hence the elution schedule shown in Figs. 1, 8, 12, 13 is preferred.

Enzymic assays. Aldolase was assayed as described by Sibley and Lehninger (1949), adenylic deaminase as by Perry and Zydowo (1959a, b), lactic dehydrogenase (LDH), phosphopyruvic kinase, myokinase, and ATPase as by Mendicino and Muntz (1958), and cathepsin as by Lapresle and Webb (1962), this last procedure modified by measuring the increase in optical density at 280 mμ of the supernatant after addition of trichloracetic acid. Enzyme activities are simply reported as changes in optical density (ΔE) with time per unit weight of freeze-dried sample.

RESULTS AND DISCUSSION

Chromatography of sarcoplasma. When 30 ml of water extract of beef skeletal muscle, longissimus dorsi, immediately after slaughter, containing approximately 900 mg of protein was chromatographed on cellulose phosphate, more than 14 peaks could be recognized in the chromatogram (Table 1, Fig. 1) (11 major fractions, Fraction I–XI in Fig. 1). When the sarcoplasmas from rabbit and beef skeletal muscle were chromatographed immediately after slaughter on DEAE-cellulose as reported by Mandeles (1960), six major fractions were recognized by Fujimaki *et al.* (1961) and seven fractions by Mandeles (1960). Even under very improved conditions carrying out the fractionation on DEAE-cellulose at pH 9.3, sarcoplasma from rabbit skeletal

Table 1. Properties of water extract of beef skeletal muscle, longissimus dorsi; chromatograph shown in Fig. 1.

Sample	pH value of extract[a]	Quantity of extract[a]	Optical density after 1:100 dilution				N content mg/ml of extract after dialysis
			Before dialysis		After dialysis		
			280 mμ	260 mμ	280 mμ	260 mμ	
Immed. after slaughter	6.15	99 ml	0.548	0.685	0.360	0.305	5.04
Freeze-dried extract	.....	.....	.....	.....	0.258	0.218	3.16
Freeze-dried stored extract[b]	.....	.....	.....	.....	0.248	0.224	.....
Heated extract[c]	.....	.....	.....	.....	0.305	0.273	3.78

[a] Addition of water 100 ml to 100 g muscle.
[b] 4.5 months at −15°C.
[c] 50°C for 30 min.

muscle could be eluted in only six major fractions by stepwise increases in the ionic strength, although at least 15 migrating bands have been recognized on starch-gel electrophoresis of the muscle by Hartshorne and Perry (1962).

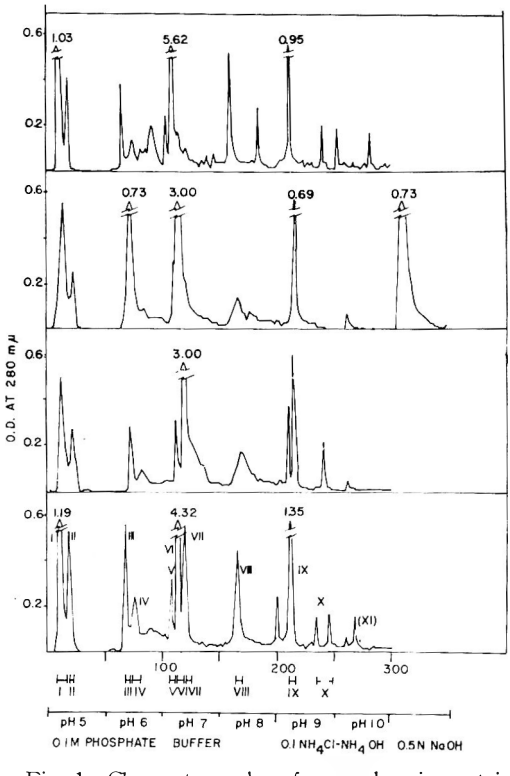

Fig. 1. Chromatography of sarcoplasmic protein of beef skeletal muscle (longissimus dorsi) on cellulose phosphate. Curves from bottom to top are: extract immediately after slaughter; freeze-dried extract; freeze-dried stored extract; and extract immediately after slaughter and heated 30 min at 50°C.

Identification of certain fractions. Fraction I (breakthrough fraction), eluted at 0.1*M* phosphate buffer solution at pH 5, differed from other fractions in possessing an optical density higher at 260 mμ than at 280 mμ (Fig. 2, Table 4). When freeze-dried preparation of this fraction was hydrolyzed with KOH and rechromatographed on Dowex-1, X-8 (formate form), fractions considered to be nucleotides (including inosine monophosphate) could be obtained by eluting with various concentrations of formic acid-formate solutions. As shown in Fig. 2, the minimum of the ultraviolet absorption curve was in the region of 245 mμ rather than at 230 mμ, the minimum characteristic of pure ribonucleic acid, which might suggest that small amounts of protein were present (Perry and Zydowo, 1959). This fraction may therefore be considered to be a nucleoprotein, but detailed elucidation on its properties will appear in a subsequent paper.

Fractions IV and VI were respectively found to correspond to oxymyoglobin (λ_{max} 542 and 580 mμ) and metmyoglobin (λ_{max} 505 and 630 mμ). Protein eluted at Fraction IX may be a denatured metmyoglobin derivative.

Mandeles (1960) reported that, although each of the activities of phosphorylase, phosphoglucose isomerase, aldolase, and adenylate kinase was present in the original extract of chicken muscle, only adenylate kinase activity could be found after chromatography, when assays for the above enzyme activities were performed on one tube from each of the peaks obtained.

Aldolase activity was associated with Fractions III through IX, although the activity decreased in Fractions IV and V (Table 2).

Figs. 3, 4, 5, and 6 show results of assays for lactic dehydrogenase, phosphopyruvic kinase, myokinase, ATP-ase, and cathepsin activities.

Lactic dehydrogenase (LDH), like aldolase, was found to be localized in the region between Fractions II and IX. In LDH, two basically different molecular forms of the H-enzyme and M-enzyme have been reported (Wilson *et al.*, 1963). As revealed in Table 3, LDH in Fractions VII and VIII was considered to be the same M-enzyme, from the degree of substrate (pyruvate) inhibition.

Adenylic deaminase could not be found in the fractionated proteins, though it was present in the original extract.

Table 4 summarizes the chemical and enzymic properties of freeze-dried fractionated proteins. All enzymic activities, except cathepsin, were found in Fraction VI (major fraction of metmyoglobin). For more detailed fractionation, freeze-dried preparation of Fraction VI (60 mg in 20 ml

Table 2. Aldolase activity of freeze-dried preparations of fractionated sarcoplasmic protein from beef longissimus dorsi.

Fraction	Aldolase activity ΔE at 540 mμ/0.125 mg dry wt.
I	0.019
II	0.021
III	0.822
IV	0.300
V	0.278
VI	1.150
VII	1.032
VIII	0.903
IX	1.123
X	0.038

Table 3. Effect of pyruvate concentration on lactic dehydrogenase activity in fractionated sarcoplasma of longissimus dorsi (beef).

	Lactic dehydrogenase activity/1 min/0.06 mg of preparation (−ΔE at 340 mμ)			
Pyruvate (moles)	10^{-2}	3.3×10^{-3}	10^{-3}	3.3×10^{-4}
Fraction VII	0.271	0.414	0.351	0.255
Fraction VIII	0.091	0.108	0.096	0.075

Table 4. Chemical and enzymic properties of freeze-dried, fractionated sarcoplasmic protein from beef longissimus dorsi.

Fraction	pH of elution buffer	Ppt with TCA[a]	Solub. in water	OD (3mg/5ml) 280 mμ	OD (3mg/5ml) 260 mμ	Enzymatic activity: Aldolase	LDH	PPK	Myok.	ATPase	Cathep.	Remarks
I	5	±	+	0.200	0.340	−	−	−	−	−	−	Nucleoprotein?
II	5	+	+	0.321	0.265	−	+	−	−	−	−	
III	6	+	+	0.268	0.202	+	+	−	−	−	−	
IV	6	+	+	0.398	0.388	+	+	−	−	−	−	λ_{max}(mμ) 542, 580 (oxymyoglobin)
V	7	+	+	−	−	+	+	−	−	−	−	
VI	7	+	+	0.459	0.293	+	+	+	+	+	−	λ_{max}(mμ) 505, 630 (metmyoglobin)
VII	7	+	+	0.212	0.169	+	+	+	±	−	−	
VIII	8	+	±	0.370	0.321	+	+	−	−	−	−	
IX	9	+	±	0.306	0.236	+	+	+	+	±	±?	
X	9	+	±	−	−	−	−	−	−	−	−	

[a] TCA = trichloracetic acid.

of 40m*M* glycine-NaOH solution at pH 9.0) was chromatographed on DEAE-cellulose in a 2×40-cm column, and optical densities at 280 mμ of eluted fractions (volume of each fraction, 10 ml) were measured, along with assays for aldolase and LDH activities. As shown in effluent diagrams in Fig. 7, many fractions could be eluted, but aldolase was localized mainly in the region of the elution solutions B, C, and D, and LDH only in the first eluted portion with the solution B. As seen in Table 4, the enzyme activities observed are associated,

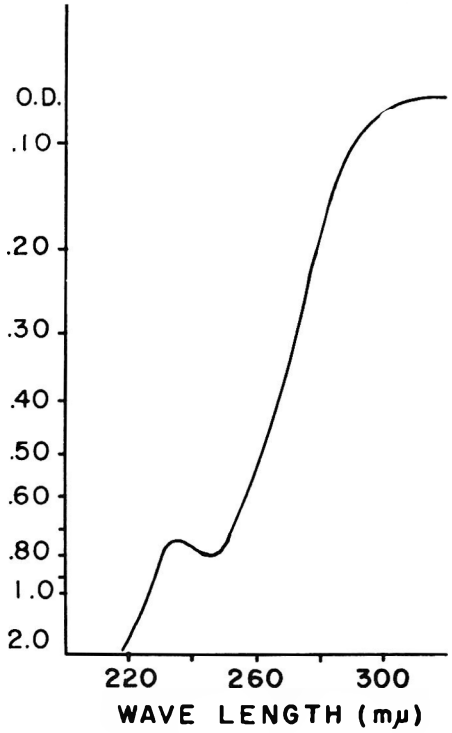

Fig. 2. Ultraviolet absorption spectrum of the peak solution of Fraction I.

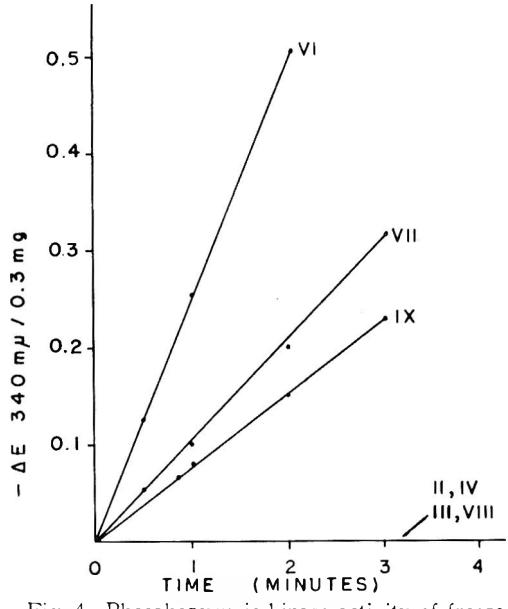

Fig. 4. Phosphopyruvic kinase activity of freeze-dried fractions of beef sarcoplasmic proteins (longissimus dorsi).

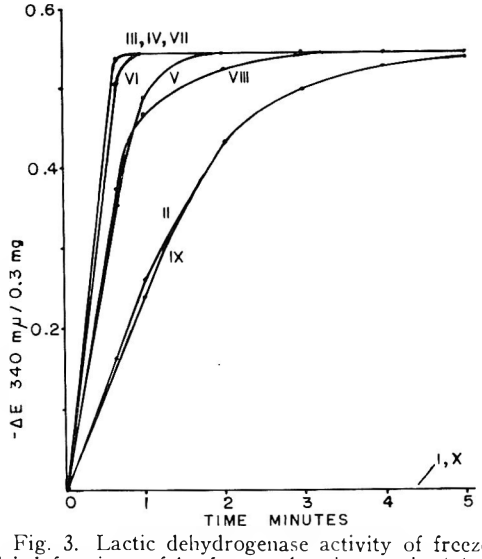

Fig. 3. Lactic dehydrogenase activity of freeze-dried fractions of beef sarcoplasmic proteins(longissimus dorsi).

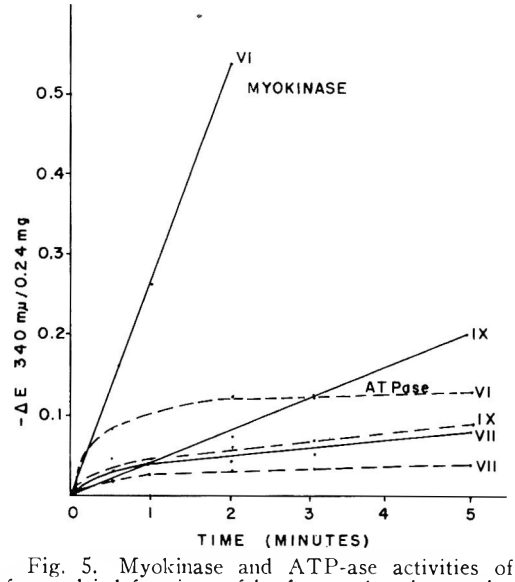

Fig. 5. Myokinase and ATP-ase activities of freeze-dried fractions of beef sarcoplasmic proteins (longissimus dorsi).

to varying degrees, with most of the major protein fractions. This may indicate that there is some protein-enzyme interaction that is not resolved on cellulose-phosphate. This explanation is supported in part by the fact that LDH can be separated on DEAE-cellulose, as shown in Fig. 7.

In previous work, protein solutions obtained at one elution peak showed two or three peaks in the ultracentrifugal analyses (Fujimaki, 1962). Consequently, fractionated protein solutions will be separated more fully by a combination of different types of ion-exchange cellulose, as mentioned above.

Effects of freeze-drying on muscle extract. Freeze-drying of meat has been shown to result in the decrease of its water-holding capacity, due to the process of dehydration (Hamm and Deatherage, 1960a). Freeze-dehydration caused some denaturtaion of muscle protein, as indicated by partial reduction of ATPase activity of actomyosin (Hunt and Matheson, 1958). Although quick freezing and thawing resulted in no considerable denaturation of muscle protein (Deatherage and Hamm, 1960), freezing of meat did decrease the extractability of sarcoplasmic proteins as well as causing a loss of specific electrophoretic and ultracentrifugal components (Kronman and Winterbottom, 1960). Moreover, electrophoresis of the sarcoplasmic proteins from freeze-dried beef showed gross denaturation during storage at elevated temperature (Cole, 1962).

As shown in Figs. 1 and 11, the eluted peaks became broader and less sharp for almost all fractions of the freeze-dried sarcoplasmic proteins. This is especially true for the two fractions VII and XI, which almost disappeared from the effluent diagrams. Even in stored freeze-dried preparation in the deep freeze, some peaks disappeared; the reason why the optical density of Fraction IV (major fraction of oxymyoglobin) increased is unknown (Fig. 1). From these results it appears that prolonged storage of freeze-dried meat may cause a loss or denaturation of some sarcoplasmic protein components.

Effects of heating on muscle extract. A strong denaturation was found to start at 40° and continue to 50°, resulting in the formation of new stable cross linkages (Hamm and Deatherage, 1960b). In muscle extract heated 30 min at 50°, most eluted peaks were recognized to increase and oxymyoglobin tended to decrease and metmyoglobin to increase (Fig. 1).

Effects of storage on muscle extract. Small differences could be recognized in the effluent diagrams between muscle extract prepared immediately after slaughter and extract stored 7 days at 1–3°, except for a decrease in the peak of Fraction I and increase in the peak of Fraction IX in the stored extract, although the pH of the latter lowered to 5.60 (Table 5, Fig. 8). Under the same conditions, the levels of eluted peaks in the effluent diagram of stored (aged) muscle have a tendency to decrease (Fig. 8), but more detailed results will appear later.

Effects of salt infusion of muscle. Muscle infused with salt solution at or soon after slaughter has been found to show a rather remarkable increase in water-holding capacity on cooking and freezing and thawing (Wierbicki *et al.*, 1954). This has suggested that the increase of water-holding

Table 5. Properties of water extract of beef longissimus dorsi; chromatograph shown in Fig. 8.

Sample	pH of extract	Quantity of extract[a] (ml)	OD after dialysis and dilution 1:100		N content of extract after dialysis (mg/ml)
			280 mμ	260 mμ	
Water extract—immed. after slaughter	6.40	65	0.315	0.288	4.93
Stored extract[b]	5.60	....	0.310	0.295	4.16
Water extract of aged muscle[b]	5.69	100	0.308	0.239	4.19

[a] Addition of water, 100 ml to 100 g muscle.
[b] 7 days at 1–3°C.

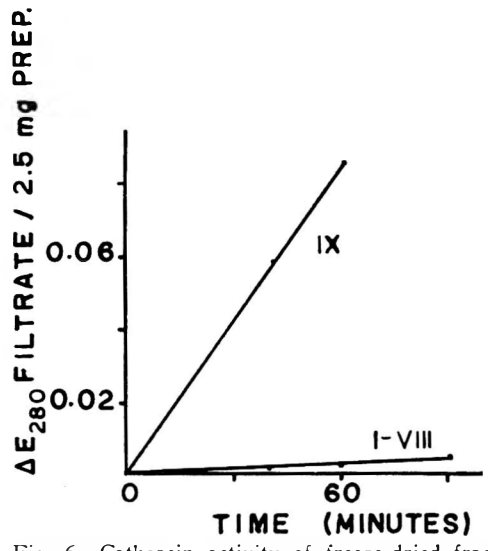

Fig. 6. Cathepsin activity of freeze-dried fractions of beef sarcoplasmic proteins (longissimus dorsi).

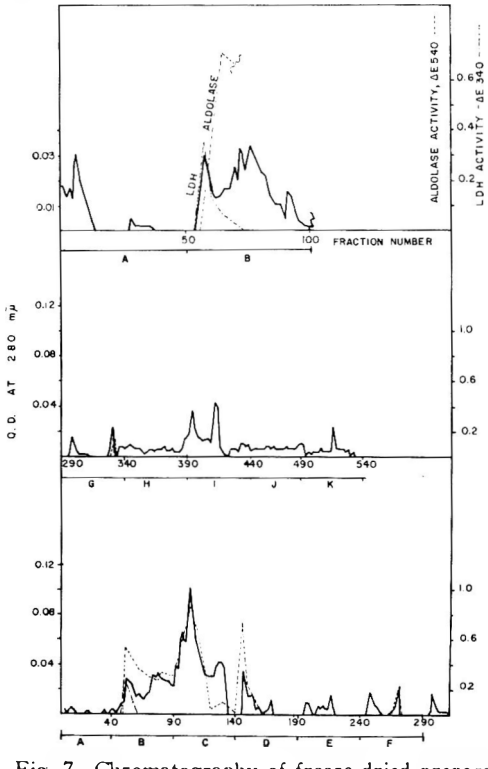

Fig. 7. Chromatography of freeze-dried preparations of Fraction VI on DEAE-cellulose, indicating a separation of aldolase and lactic dehydrogenase activities. Upper curve shows more precise separation of lactic dehydrogenase and aldolase activities in another sample of Fraction VI. Elution buffers were as follows: A) 40m*M* glycine–NaOH to pH 9; B) A + 0.01*M* KCl; C) A + 0.03*M* KCl; D) A + 0.06*M* KCl; E) A + 0.1*M* KCl; F) A + 0.2*M* KCl; G) A + 0.5*M* KCl; H) A + 1.0*M* KCl; I) 40m*M* phosphate + 1.0*M* KCl, pH 8.0; J) same as *I* except pH 7.0; K) same as *I* except pH 6.0.

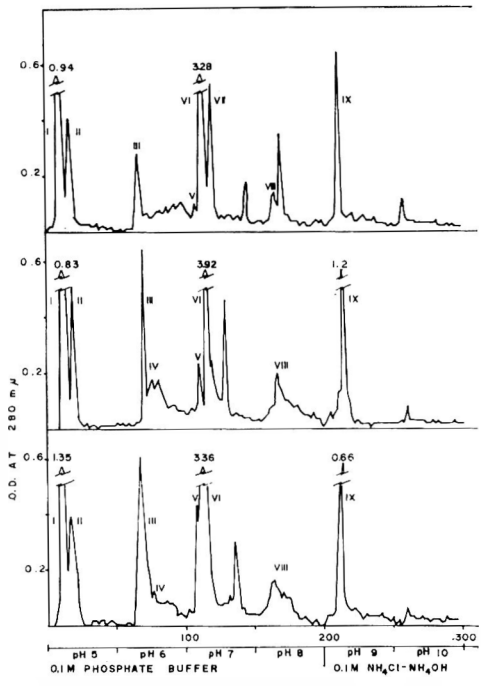

Fig. 8. Chromatography of beef sarcoplasmic proteins (longissimus dorsi) on cellulose phosphate. Curves from bottom to top are: extract immediately after slaughter; extract stored 7 days before chromatographing; and same muscle aged 7 days before extraction and chromatographing.

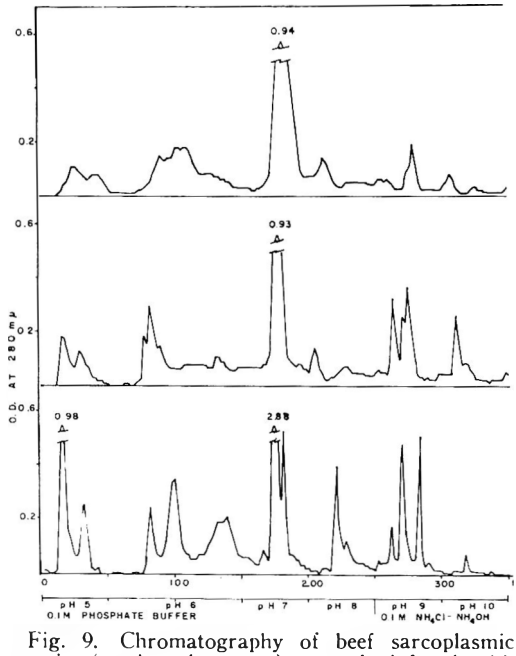

Fig. 9. Chromatography of beef sarcoplasmic protein (semimembranosus); muscle infused with 10% sodium chloride to a 7% increase in weight. Curves from bottom to top are: extract of muscle 1 day post-mortem; 12 days post-mortem; and freeze-dried extract of same muscle 12 days post-mortem.

Table 6. Properties of water extract of beef semimembranosus; chromatograms shown in Figs. 9, 10, 11.

Sample	Aging (day)[a]	pH of extract	Quantity (ml) of extract obtained[b]		OD of extract after dialysis[c]		N content (mg/ml) of extract after dialysis
			Before dialysis	After dialysis	280 mμ	260 mμ	
Muscle, infused[d]	1	5.89	42	48	0.361	0.294	5.15
Muscle, infused	12	6.02	26	35	0.286	0.184	3.78
Muscle, salt extracted	1	5.71	53	61	0.306	0.245	4.07
Muscle, salt extracted	12	5.90	44	56	0.288	0.185	3.75
Muscle, control	1	5.71	56	64	0.294	0.238	3.88
Muscle, control	12	5.85	41	55	0.260	0.170	3.44

[a] 2–4°C.

[b] Addition of water for extraction 50 ml, muscle/water = 1/1. Actual composition of extractions in these experiments: Muscle infused 53.5 g and 46.5 g water; muscle, salt extracted, 50.0 g muscle, 3.5 g 10% NaCl and 46.5 g water; muscle, control 50 g and 50 g water.

[c] Extract diluted 1:100.

[d] 10% NaCl solution at 37°C; 7% weight of muscle.

capacity in meat is attributed to some change in the nature of ion-protein interaction.

As shown in Table 6 and Figs. 9, 10, and 11, effluent diagrams from the water extract of infused muscle were compared with those from the water extract of the paired muscle of the same animal without infusion and those from salt extract of the same uninfused muscle. In the latter instance, NaCl in the extracting solution was adjusted to equal the NaCl in water extract of the infused muscle. No significant differences could be recognized between infused muscle and the control except a decrease in the level eluted at Fraction VIII. If this be true, changes of water-holding capacity in meat may be ascribed to the myofibrillar rather than the sarcoplasmic protein.

Effects of storage (aging) of muscle. During aging of meat, little changes of sarcoplasmic proteins could be found from results of conventional electrophoretical analyses (Fujimaki and Nakajima, 1958).

It has recently been shown, by the use of vertical starch-gel electrophoresis, that certain sarcoplasmic proteins are unstable post-mortem and that minor components are denatured and become immobile at the normal ultimate pH of muscle at 5.5, even when the post-mortem pH drop is slow, but many of the protein constituents are completely stable under the condition of low ultimate pH and/or a fast rate of pH fall (Scopes and Lawrie, 1963). When sarcoplasmic proteins from water extracts of muscle during aging were fractionated on cellulose-phosphate at pH 5, some eluted fractions disappeared and some diminished from the effluent diagrams (Tables 6, 7; Figs. 9, 10, 11, 12, 13). These results support the hypothesis that sarcoplasmic proteins are not completely stable during the aging of meat, because of denaturation of protein. The properties of these fractionated proteins are currently under investigation.

Although inspection of the chromatograms of Figs. 12 and 13 indicates some possible differences between the sarcoplasmic proteins of beef and veal, further work is needed to characterize these differences fully. As would be expected, there are more similarities than differences in beef and

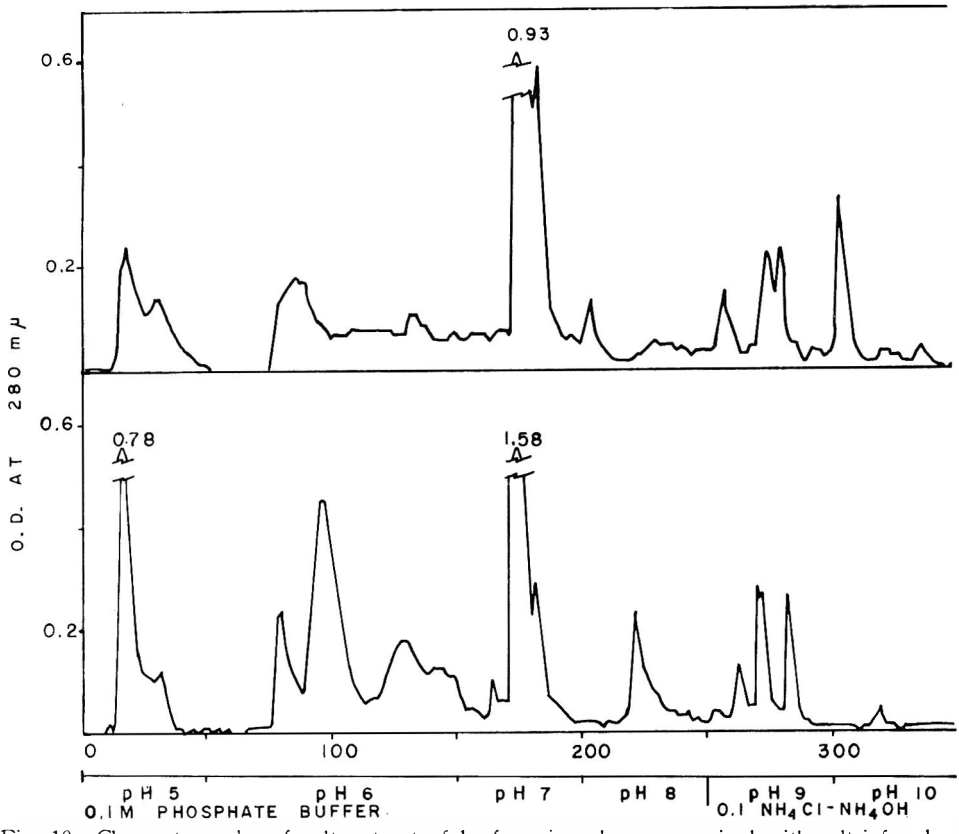

Fig. 10. Chromatography of salt extract of beef semimembranosus paired with salt-infused muscle in Fig. 9. Bottom curve is from muscle 1 day post-mortem, and top curve is from 12 days post-mortem.

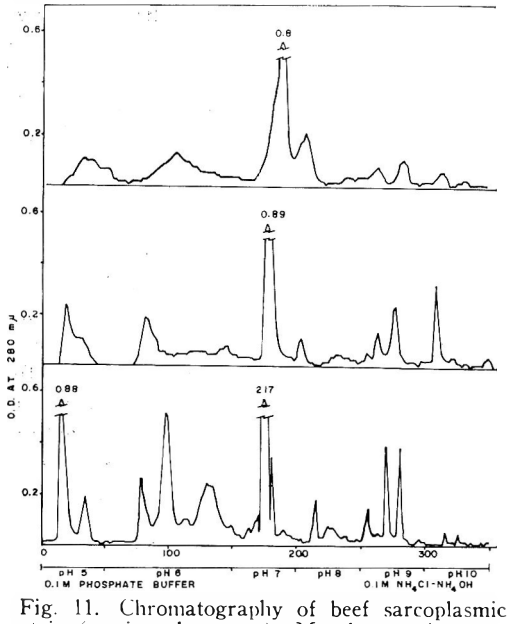

Fig. 11. Chromatography of beef sarcoplasmic protein (semimembranosus). Muscle was the same as salt-extracted muscle in Fig. 10 and also represents control for infused muscle as in Fig. 9. Curves from bottom to top are: 1 day post-mortem; 12 days post-mortem; freeze-dried extract of muscle 12 days post-mortem.

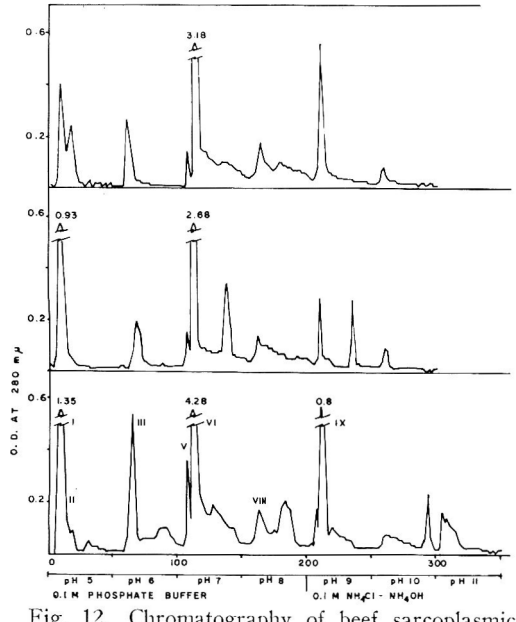

Fig. 12. Chromatography of beef sarcoplasmic protein (longissimus dorsi). Curves from bottom to top are: immediately after slaughter; 1 day post-mortem; and 7 days post-mortem.

Table 7. Changes of properties of water extract of beef longissimus dorsi during aging at 1-3°C.

	Days aged	pH of extract	Quantity of extract[a] (ml)	OD of extract after dialysis and dilution 1:100 at 280 mμ	N content of extract after dialysis (mg/ml)
Beef	0	6.50	58	0.388	4.98
	1	5.58	112	0.300	4.21
	7	5.50	100	0.286	3.93
Veal	1	5.60	110	0.360	4.35
	7	5.55	103	0.315	3.84
	14	5.52	92	0.347	3.95

[a] Addition of water, 100 ml to 100 g muscle.

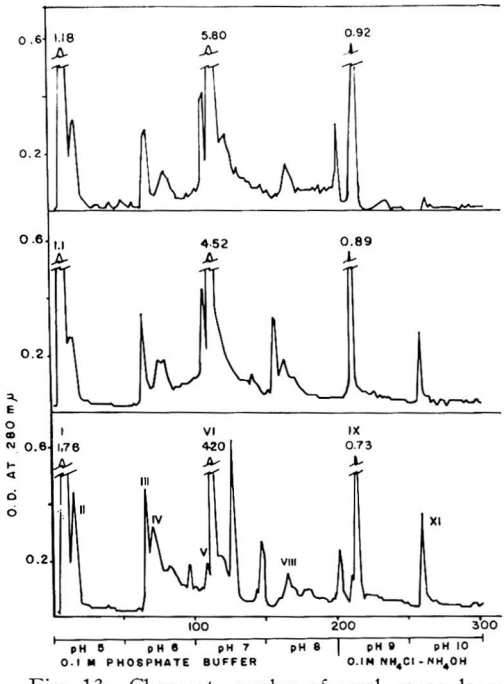

Fig. 13. Chromatography of veal sarcoplasmic protein (longissimus dorsi). Curves from bottom to top are: 1 day post-mortem; 7 days post-mortem; and 14 days post-mortem.

veal longissimus dorsi. As we compare two beef animals (Figs. 1 and 12) there appear to be quantitative differences between animals and/or muscles (longissimus dorsi and semimembranosus, Figs. 1 and 11) even though the qualitative similarities are quite clear. Much work will be needed to elucidate the significance of any quantitative differences reported here.

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Effect of Lipid Content on Protein-Sodium Linolenate Interaction in Fish Muscle Homogenates

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SUMMARY

A species difference in reactivity of fish muscle proteins toward sodium linolenate in muscle homogenates is demonstrated. The proteins of muscle having higher lipid content show greater stability. Sodium linolenate uptake by natural lipids, resulting in competition between lipids and protein for added sodium linolenate, is suggested. The significance of these findings in terms of a possible *in situ* relationship of lipid hydrolysis to protein denaturation in frozen stored fish muscle is discussed.

INTRODUCTION

Dyer and associates (Dyer and Dingle, 1961) showed that lipid degradation may be involved in frozen-storage-induced denaturation of fish muscle proteins. Dyer found greater resistance to denaturation in the more fatty fishes—plaice, halibut, ocean perch—than in lean fish such as cod. He explained this by postulating that either: 1) the lipid associated with the protein stabilizes the protein molecule against denaturation, and this protection is lost when the lipid is hydrolyzed; or 2) fatty acids liberated from the lipids on hydrolysis modify the surface of the protein molecule, making it subject to denaturation.

Olley *et al.* (1962) tested Dyer's hypotheses on cod, halibut, lemon sole, and spiny dogfish. They found no correlation between protein stability and remaining unhydrolyzed lipid during storage of these species at −14°C. They also found that in the presence of similar quantities of fatty acid released *in situ* during storage, the protein of halibut and lemon sole was more stable than that of cod, using solubility in 5% neutral salt solution as the criterion. In the dogfish, moreover, much larger amounts of fatty acid were liberated, mostly from triglycerides (in other species phospholipids are the principal source of fatty acids), yet dogfish proteins exhibited a stability slightly greater than that of cod. They argued, therefore, that there was no simple causal relationship between phospholipid breakdown and/or free fatty acid production and protein denaturation in fish muscle.

We have reported that C_{18} unsaturated fatty acids insolubilize cod actomyosin in muscle extracts (King *et al.*, 1962). The results of Dyer and Olley suggest that the natural fish lipids might influence fatty acid–protein interaction in model systems. We have extended our studies of the reactivity of fish muscle proteins toward fatty acid to species other than cod in order to throw light on a possible interrelationship of natural lipid content, lipid degradation, and protein denaturation.

In the present study we investigated the effect of added fatty acid on protein solubility in seven species of fish. The species studied were chosen for their range of lipid content: lean fish, cod and haddock; somewhat more fatty fish, flounder and ocean perch; fatty fish, mackerel. The elasmobranch spiny dogfish was included as a species having high lipid content but poor protein stability. The skate was chosen as an elasmobranch of relatively low lipid content. A relationship between lipid content and protein-insolubilization resulting from protein–fatty acid interaction was sought. During the investigation of the effect of fatty acids on dogfish proteins, we noticed in the extracts the presence of a low-density insoluble material which had considerable effect on the stability of dogfish proteins. Since this material was largely lipid in character, its relationship to protein–fatty acid interaction was investigated.

EFFECT OF LIPID CONTENT ON PROTEIN INSOLUBILIZATION

Materials and methods. *Materials.* Cod (*Gadus morhua*, also called *G. callarias*, Cohen, 1959), haddock (*Melanogrammus aeglefinus*), winter or blackback flounder (*Pseudopleuronectes americanus*), mackerel (*Scomber scombrus*), the big

skate (*Raja ocellata*), spiny dogfish (*Squalus acanthias*), and ocean perch (*Sebastes marinus*) were obtained commercially. When obtained, they had been stored in ice for not more than 1 day after capture.

The linolenic acid and the buffered extractant used in this study have been described in a previous paper (Anderson *et al.*, 1963).

Muscle tissue sampling. Two to six fish of each species were used for tissue sampling. Interior sections, free from adipose tissue on visual examination, were cut from the anterior portion of the fillets of the teleosts and dogfish and the pectoral muscle of the skates. The sections were diced and mixed so that representative portions of tissue could be used for both lipid and protein extractions.

Preparation of sodium linolenate. Aqueous solutions of the sodium salt of linolenic acid were prepared according to the method of Goodman (1958).

Preparation of protein extracts. Muscle-extractant homogenates were prepared in the manner previously described for cod (Anderson *et al.*, 1963) except that the tissue-extractant ratio was adjusted to give a final ratio of 10:400 (15:400 in the case of skate) after the addition of sodium linolenate solution and double-strength extractant (required to maintain ionic strength).

Addition of sodium linolenate solution to portions of muscle-extractant homogenates. To a series of 390-ml portions of homogenate were added 0–10 ml of a solution of sodium linolenate, 10 ml of double-strength extractant, and enough distilled water to make a total volume of 410 ml. The portions were then blended as previously described.

Preparation and treatment of homogenates was carried out in a 4°C refrigerated room using precooled glassware.

Extraction of lipids. For each species, three 10-g samples of tissue were extracted and washed by the method of Folch (1957).

Analytical procedures. Soluble protein nitrogen content was determined as previously described (Anderson *et al.*, 1963). Total lipid content was determined on each lipid extract.

Results and discussion. Fig. 1 shows changes in soluble protein content of muscle extracts treated with sodium linolenate. The curves show that there is little or no loss in soluble protein until a critical concentration of sodium linolenate is reached. Beyond that point, the soluble protein content fails rapidly with only slight increases in sodium linolenate concentration. The initial plateau area and the critical concentration of sodium linolenate are different for each species.

Fig. 2 shows lipids plotted against sodium linolenate concentration at which half of the protein loss is sustained. It shows linearity between

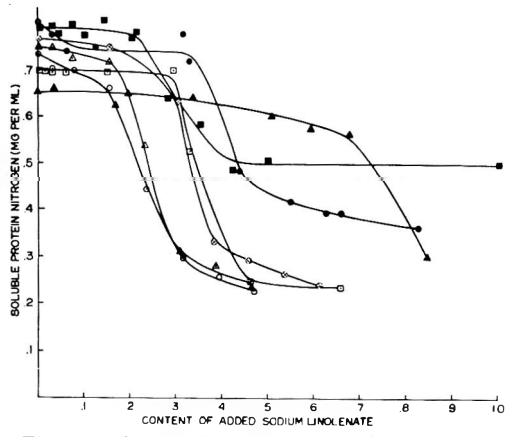

Fig. 1. Change in soluble protein content of muscle extracts after addition of sodium linolenate solution. The concentration of added sodium linolenate is expressed as the ratio of mg of fatty acid salt, calculated as linolenic acid, to mg of soluble protein nitrogen in control samples. ⊙—⊙, cod; △—△, haddock; ⊡—⊡, flounder; ⊗—⊗, ocean perch; ■—■, skate; ●—●, mackerel; ▲—▲, dogfish.

total lipid content and reactivity of muscle proteins toward linolenate in cod, haddock, skate, flounder, and ocean perch. The points for dogfish and mackerel, which species have relatively high lipid contents, are not linearly related to the points for the leaner species. Because other "fatty" species were not available, it could not be determined if there is a linear relationship between total lipid content and reactivity of muscle proteins toward linolenate in "fatty" fish. Differences in lipid composition and lipid dispersibility in the homogenate are sufficient to explain why this relationship need not be linear.

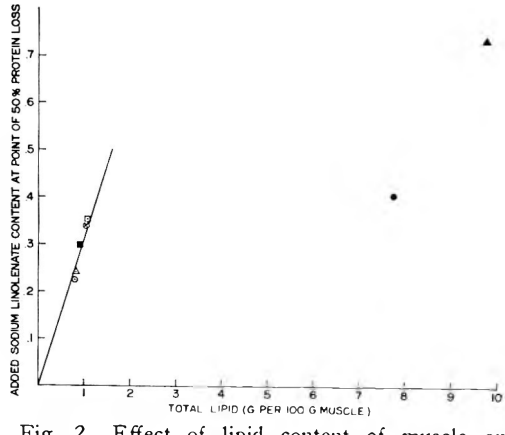

Fig. 2. Effect of lipid content of muscle on protein-sodium linolenate interaction. Values of sodium linolenate content are taken from curves in Fig. 1. ⊙, cod; △, haddock; ⊡, flounder; ⊗, ocean perch; ■, skate; ●, mackerel; ▲, dogfish.

These results suggest a protective effect on the part of the natural lipids resulting from competition between lipids and proteins for added fatty acid. Affinity of lipids for lipid-like substances is well known. In mammalian plasma, for example, lipoproteins act as carriers for fatty acids and other lipid substances. Lipids are thought to be bound to, or dissolved or emulsified in, the lipid carrier, or held by van der Waals forces (Chargaff, 1944; Neurath and Bailey, 1954).

EFFECT OF LOW-DENSITY INSOLUBLE MATERIAL ON REACTIVITY OF PROTEINS IN DOGFISH EXTRACTS

Some insight into the way in which lipids may influence protein-sodium linolenate interaction was obtained from a set of experiments with dogfish tissue extracts. When dogfish muscle is blended with extractant, an opaque, insoluble material rises to the surface of the extract. The material has a high-lipid low-protein content. A satisfactory analysis has not been obtained, since disruption of its structure occurred when attempts were made to free it of associated muscle protein. This suggests that the material is either a denatured lipoprotein or an oil-in-water emulsion stabilized by protein. The effect of this material on protein-sodium linolenate interaction in dogfish extracts was determined.

Methods. Muscle extractant homogenate was prepared as in the first experiment. It was transferred to an aspirator bottle and mixed by a magnetic stirrer for 10 min. Stirring was continued while three equal volumes of homogenate were drawn off. The insoluble low-density material of sample no. 1 was concentrated by centrifugation at 10,000 × G, removed somewhat incompletely from the surface, and added to sample no. 2. Sample no. 3 served as control. All three samples were then mixed by magnetic stirring and then further divided into portions for teratment with sodium linolenate solution. Addition of sodium linolenate was carried out as in the first experiment except that mixing was accomplished with a multiple-unit magnetic stirrer rather than by blending.

Results and discussion. Small amounts of this low-density insoluble material were found to have a substantial effect on protein sodium-linolenate interaction in dogfish extracts. Fig. 3 shows clearly that partial removal of the material from an extract results in increased insolubilization of muscle protein in the presence of sodium linolenate. Addition of the material to extracts containing the usual amount of this material decreased the amount of protein insolubilized by sodium linolenate. The results suggest uptake of sodium linolenate by this material, and a greater affinity of sodium linolenate for this material than for muscle protein under these conditions.

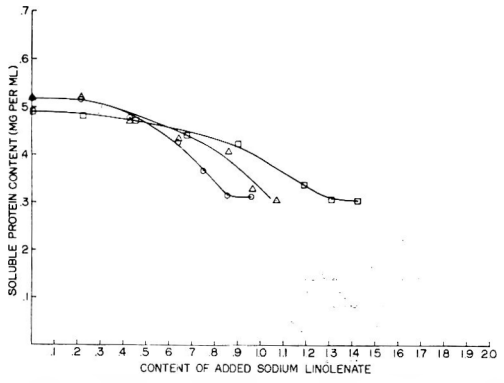

Fig. 3. Effect of low density, opaque material on protein-sodium linolenate interaction in dogfish homogenates. The concentration of added sodium linolenate is expressed as the ratio of mg of fatty acid salt, calculated as linolenic acid, to mg of soluble protein nitrogen in control samples. ⊙—⊙, Extract I, material removed from extract; ⊡—⊡, Extract II, material added to extract; △—△, Extract III, control.

This experiment demonstrated clearly that natural lipids can influence protein-sodium linolenate interaction in a model system. If the significance of these findings is to be related to the influence of lipid hydrolysis on protein denaturation in the frozen-stored muscle tissue itself, it will be necessary to seek a relationship between the amount of fatty acid liberated, the amount of lipid left unhydrolyzed, and the extent of protein denaturation. In an estimation of lipids involved in the process, lipid distribution at the subcellular, cellular, and interstitial levels will have to be considered. Of importance, too, will be information concerning the fatty acid uptake capacities of lipids involved and the degree of saturation and chain length of fatty acids liberated. Species differences in content and kind of sarcoplasmic proteins and possible differences in the affinity of these proteins, as well as myofibrillar proteins, for fatty acids may also be factors in determining the relationship of lipid degradation to protein denaturation.

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Certain Microbial Indices of Frozen Uncooked Fish Fillets[a]

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SUMMARY

Frozen fish fillets from commercial outlets representing 26 producers were analyzed for total aerobic plate counts, coliform organisms, fecal streptococci, and coagulase-positive staphylococci. Total plate counts ranged from 1300 to 1,800,000/g; 72% were below 200,000/g. Coliform counts were low (0–190/g). Only 14 of the 78 samples contained coliform organisms. Presumptive fecal streptococci counts were usually appreciably higher than coliform counts, and in only a few samples were none found. The highest count noted was 19,000 organisms/g. Coagulase-positive staphylococci were present in 25 samples, representing 17 producers. Unlike in a previous study on frozen shrimp, half of the egg-yolk-positive micrococci were not coagulase-positive.

INTRODUCTION

In microbial examination of frozen foods, processing and storage variables result in progressive changes in microbial survival patterns. The use of microbial indices for evaluation of a processed food sampled during storage implies that the detected organisms have either public-health or economic significance. Elliott and Michener (1960, 1961) have reviewed this subject, and Levine (1961) suggested a limit for a standard plate count of 100,000 organisms per g capable of growth at 35–37°C. This recommended temperature of incubation is based on the growth characteristics of those organisms of main concern in public health and minimizes the significance of psychrophilic and other food-spoilage organisms that have been implicated in microbial deterioration of products stored at refrigeration temperatures. Levine indicates that the standard plate count can serve as a practical index for evaluating sanitary practices.

In this regard, Canada (Anderson, 1962) has tentatively established a maximum allowable limit of 250,000 viable organisms per g at 25°C as a means of enforcing sanitation standards in the fishing industry. This ruling has succeeded in lowering the initial median count per g of processed frozen fish fillets in processing plants from 260,000 in 1959 to 66,000 in 1962.

In previous studies (Silverman *et al.*, 1961a,b; Nickerson *et al.*, 1962) the products examined were, for the most part, cooked or partially cooked shrimp or fish fillets, and the coliform index was suggestive of contamination occurring in certain of the products after heat treatment. The coliforms are not notably resistant to frozen storage, and for processed fresh and frozen poultry and chicken pies the enterococci content is considered by many to be a better indication of unsanitary conditions (Wilkerson *et al.*, 1961; Hartman, 1960). Estimation of enterococci by the most probable number, although found to be satisfactory (Raj *et al.*, 1961), is somewhat cumbersome for evaluation of a large number of samples.

Kenner *et al.* (1960, 1961) proposed the use of a convenient plating medium designated KF streptococcal medium. Since the *Streptococcus salivarius, S. bovis, S. equinus,* enterococcus biotypes groups, in addition to the enterococcus group (D), are present in varying amounts in animal feces, they suggested the use of the more general term "fecal streptococci." This is an extension of the more conventional employment

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of group D enterococci (Sherman, 1937). The designation "fecal streptococci" may be misleading in that these organisms may, in certain cases, have originated from nonfecal sources (Thatcher, 1955; Mundt, 1961, 1963).

It was therefore thought to be of interest to compare the standard plate count to the incidence of coliform, fecal streptococci, and coagulase-positive staphylococci in frozen raw fish fillets.

MATERIALS AND METHODS

Three samples, from each of 26 producers, consisting of raw and frozen fillets of cod, haddock, and ocean perch were obtained from retail outlets across the country and shipped to the laboratory packed in dry ice. The samples were stored at −40°C until analyzed.

The samples were segmented while still frozen, and representative portions totaling 50 g were comminuted with 450 ml of diluent in a blender for 2 min and serial dilutions made for microbial analysis. The diluent consisted of 0.1% trypticase (BBL) adjusted to pH 7.0. Standard aerobic plate counts were made in plate count agar (Difco Lab., Inc.) incubated 48 hr at 30°C.

Coliform organisms were analyzed by methods previously used (Silverman *et al.*, 1961a). Representative isolates from desoxycholate-lactose agar (Difco Lab., Inc.) were inoculated into brilliant green bile broth. Those cultures which were typical gram-negative lactose fermenters were streaked on eosin methylene blue (EMB) agar for colony characterization.

Fecal streptococci were enumerated with the KF medium of Kenner *et al.* (1961). In our initial studies their medium was formulated in this laboratory, but in subsequent studies involving confirmation, a commercial source (Difco Lab., Inc.) was used. Colonies from KF agar medium were streaked on brain heart infusion agar and, after verification of purity, stored at 20°C. Physiological tests were in accord with the procedures of Sherman (1937) and Kenner *et al.* (1960).

Coagulase-positive staphylococci were detected on egg yolk (EY) agar after preincubation in cooked meat medium containing 10% sodium chloride (Carter, 1960; Silverman *et al.*, 1961b). For attempted enumeration directly on agar plates, the medium of Innes (1960) (IM) was modified by substituting egg-yolk (1 yolk per liter) in place of Oxoid egg yolk emulsion and adjusting the sodium chloride content to either 1 or 7.5%. EY agar containing 10% sodium chloride (EY-10) was the other medium tested. Coagulase was determined with Difco coagulase plasma on EY-positive colonies grown 24 hr at 37°C in brain-heart infusion broth.

RESULTS AND DISCUSSION

The standard plate count ranged from 1300 (producer 12) to 1,800,000 organisms per g (producer 24) (Table 1). Approximately 73% of the samples had a standard plate count of 200,000 or less. Although these counts are, in general, low, organoleptic evaluation indicated that deteriorative changes have occurred (Nickerson and Licciardello, 1963). Samples having counts in excess of 200,000 were distributed among 14 of the 26 processors. The products of certain of the processors were consistently high in bacterial count, notably nos. 17, 20, 24, and 26, which had, out of three samples, either two or three samples with a count in excess of 200,000 organisms per g. Ocean perch was generally higher in bacterial count than either cod or haddock (Fig. 1). Of 27 samples of cod and haddock and 24 samples of ocean perch, 22 cod, 23 haddock, and 11 ocean perch samples had a plate count of less than 200,000. Of the total of 78 samples, 20 were in excess of 250,000 organisms per g, and these were distributed among 13 producers.

Only 14 of the 78 samples were found to contain coliform organisms. The highest coliform count was 190 per g, with only four samples having 10 or higher. The presumptive fecal streptococci counts were greater than the coliform counts. In 9 samples streptococci were absent, and one sample was found to contain 19,000 fecal streptococci per g (producer 19). Thirty-six of the samples (16 producers) had counts of over 100 per g. It is apparent from Fig. 2 that there is a general agreement between coliform, fecal streptococci, and standard plate count, especially at higher levels of microbial contamination, but the low incidence of coliforms appears to negate its value as an indicator of sanitary quality for uncooked frozen fish fillets sampled during frozen storage.

The lower incidence of coliforms than of fecal streptococci detected in this study is not surprising. The samples were obtained from commercial sources after an indeterminate period of frozen storage. The coli-

Table 1. Microbial analysis of frozen fish fillets.

Source	Producer	Total count (organisms per g)	Coliform organisms per g	Fecal streptococci per g (presumptive)	Coagulase-positive staphylococci [a]
Cod	1	1,500– 37,000	0–6	10–75	1/3
	2	27,000– 350,000	0	90–200	1/3
	3	13,000– 38,000	0	5–550	1/3
	4	12,000– 600,000	0–2	85–7,300	1/3
	5	16,000– 45,000	0	5–35	1/3
	6	180,000– 870,000	0	250–900	2/3
	7	20,000– 44,000	0	10–130	0/3
	8	68,000– 480,000	0	25–210	1/3
	9	15,000– 120,000	0	0–10	1/3
Haddock	10	10,000– 27,000	0	0–15	2/3
	11	7,000– 84,000	0	15–35	1/3
	12	1,300– 130,000	0	0–15	0/3
	13	96,000– 120,000	0	5–1,500	0/3
	14	1,500– 260,000	0	10–25	0/3
	15	1,400– 3,600	0	5–10	0/3
	16	74,000– 260,000	0–45	170–210	2/3
	17	25,000– 410,000	0	5–140	0/3
	18	9,000– 15,000	0–1	0–20	0/3
Ocean perch					
	19	72,000– 820,000	0–11	220–19,000	1/3
	20	300,000– 440,000	0	0–2,000	1/3
	21	38,000– 460,000	0–190	370–3,600	3/3
	22	43,000– 320,000	0–10	0–685	1/3
	23	46,000– 200,000	0	150–1,000	0/3
	24	220,000–1,800,000	0–5	500–12,000	0/3
	25	4,100– 130,000	0–15	80–760	3/3
	26	110,000– 680,000	2–7	400–3,700	2/3

[a] Number of samples containing coagulase-positive staphylococci in three samples analyzed.

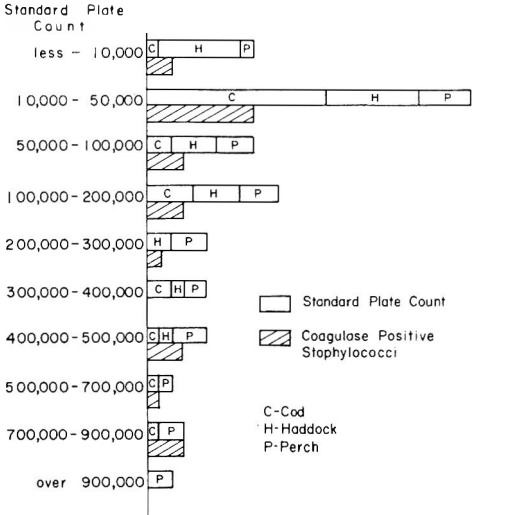

Fig. 1. Standard plate count of frozen fish fillets and the incidence of coagulase-positive staphylococci.

forms are much more susceptible than the streptococci to freezing and frozen storage, and *Escherichia coli*, one of the more labile of the coliform organisms, has a rate of mortality during freezing and frozen storage comparable to that of salmonellae (Kereluk and Gunderson, 1959; Elliott and Michener, 1960; Ingram, 1961). Forty-one of the coliform isolates from fillets were streaked on EMB agar for further confirmation. Of these, 58% appeared to be typical *Aerobacter* strains, 20% slow lactose fermenters, and only 22% gave a sheen typical of *Escherichia coli*. Dependence on a sheen may also cause an overestimate of the number of *E. coli* (Raj and Liston, 1961).

In the present study, 56 cultures, all catalase-negative isolates from KF medium, were examined, and 46 (82%) possessed typical group D characteristics. Examination of the remaining 9 catalase-negative cultures (Table 2) indicated that four may

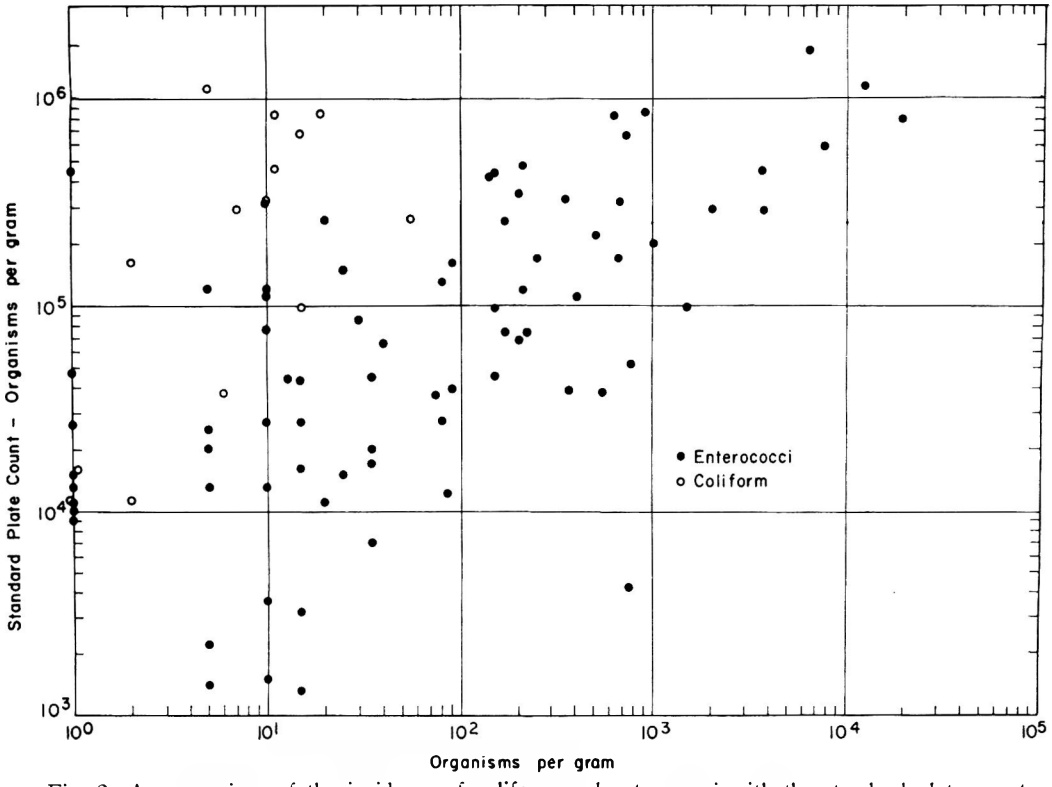

Fig. 2. A comparison of the incidence of coliform and enterococci with the standard plate count.

Table 2. Growth in various test media of nine isolates from KF medium which do not conform to Group D enterococci.[a]

Ability to reduce litmus milk	pH 9.6	6.5% NaCl	45°C
−	+	−	+
−	+	−	sl
−	−	−	sl
+	+	−	−
sl	+	sl	−
sl	+	+	−
−	+	−	+
−	+	−	+
sl	+	+	−

[a] + growth; − no growth; sl slight growth.

be biotypes. There is also a possibility that certain of these are cultures of the azide-tolerant species and catalase-negative strains of the genus *Aerococcus* (Diebel and Niven, 1960), although four of six strains tested by these investigators had been found to be catalase-positive. The remaining culture isolated from KF medium was a catalase-negative rod. Equivalent recovery was obtained for all of the 56 isolates plated in plate count and KF agars. KF medium appeared to be a selective but noninhibitory medium for the purpose it was intended, but the small number of isolates examined prevents further conclusions.

Table 1 and Fig. 1 show that the total plate count is a poor indicator of whether or not coagulase-positive staphylococci are present. These organisms were present in the products of 17 of the 26 producers examined, 6 of which had two positive samples from each of the three analyzed. For two plants (producers 21 and 25) that processed perch, all three samples were positive. The method employed here was not quantitative, and indications are that the organisms were present in low numbers.

In a previous study on shrimp, Silverman *et al.* (1961b) noted that coagulase-positive staphylococci were detected in 75% of the shrimp samples and that the improvement in detection, although qualitative, was due to the improved recovery procedure. The procedure involved incubation in cooked meat medium containing 10% sodium chlo-

ride and subsequent plating on EY agar. Although not all EY-positive cultures were coagulase-positive staphylococci, the presence of halos greatly aided in their selection. Jay (1961) also considered EY agar as the best of several selective media used. In the present study with frozen raw fish fillets, those colonies having definite and extended halos on EY agar were generally coagulase- and catalase-positive cocci. The correlation between the presence of halos and the possession of coagulase for isolates from fillets was not as good as previously experienced for isolates from shrimp, about 50% of the haloed colonies isolated from fillets being coagulase-positive. A number of weak egg yolk reactions were noted, where the lipase reaction responsible for the halo (Shah and Wilson, 1963) was limited to the area below the colony, and detectable only when the colony was lifted from the agar. Very few of these isolates possessed coagulase. Attempts failed to find a direct plating medium which would be selective for coagulase-positive staphylococci and would also incorporate the egg yolk reaction as an indicator. Media based on IM containing 1 or 7.5% sodium chloride, and EY agar containing 10% sodium chloride (EY-10) were either nonselective or, in the case of IM with a higher chloride content, too inhibitory for the initial isolation. This may have been, in the case of the IM media with 7.5% sodium chloride, due to the combination of high salt and tellurite (Zebovitz *et al.*, 1955). In a separate experiment, isolates obtained from the cooked meat plus EY procedure and streaked on either IM or EY-10 gave definite typical black colonies and halos on IM and EY-10 agars. It is conceivable that preincubation in a nonselective nutrient medium such as cooked meat medium might result in a more successful recovery of coagulase-positive staphylococci. This preincubation procedure has been advocated for enterococci (Childs and Allen, 1953) and for salmonellae (North, 1961). Another possibility is that these organisms are too few in number to be detected without introducing an enrichment step. A new selective medium incorporating pyruvate for enhanced recovery was not tested in this investigation (Baird-Parker, 1962).

The value of the coagulase reaction in detecting *Staphylococcus aureus* has been emphasized (Evans *et al.*, 1950; Mossel, 1962; and Baird-Parker, 1963). An estimation of the number of coagulase-positive staphylococci isolated from fish fillets and capable of producing enterotoxin will be the subject of a subsequent paper.

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RESEARCH NOTE

Viability and Heat Resistance of Anaerobic Spores Held 20 Years at 40°F

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During a study of the influence of various factors upon the thermal resistance and growth of a putrefactive anaerobe in meat (Stumbo *et al.*, 1944), it was observed that the number of spores in the original stock culture remained at approximately the same level. The stock culture was stored in a refrigerator at 40°F for a period of 20 years. Spore counts made on the stock culture during storage showed the spore count has remained at approximately the same level but there has been a change in the thermal resistance.

PROCEDURE

In 1943 when this laboratory was starting to study the influence of various factors upon the thermal resistance and growth of a putrefactive anaerobe in meats, it was necessary to have a mass volume of the test bacteria so that comparable spore suspensions could be reproduced when they were needed. The putrefactive anaerobe used was isolated from spoiled cured, canned meat. The anaerobe was found to be culturally and serologically identical with PA 3679, a test organism used by NCA Laboratory and designated as strain S_2 PA 3679 (Gross *et al.*, 1946).

The medium used for producing the spores was dextrose brain broth. Medium was prepared as follows: 5 g salt (NaCl), 10 g Difco Peptone, 5 g Difco beef extract, and 5 g dextrose were dissolved in 1 L water to make a nutrient broth. This broth was then distributed in screw-cap bottles containing a few glass beads and an equal volume of chopped brains. The caps were screwed down tightly and the bottles autoclaved 30 min at 121°C. After sterilizing, the bottles were cooled and the medium inoculated with the anaerobe.

After 6 days of incubation at 37°C the bottles were removed from the incubator and shaken mechanically for 30 min. After 6 days of incubation this organism digested the brains so that they were completely emulsified during the shaking process. The emulsion was filtered through sterile glass wool into a 32-oz sterile glass bottle with screw cap. The bottle was stored in a refrigerator. Throughout the years the bottle has been removed from the refrigerator, thoroughly mixed, opened and a portion of filtrate (filtered emulsion) removed. The filtrate was used directly as inoculum and for spore counts.

A dilution method was used for spore estimation. Serial decimal dilutions of filtrate were made in sterile saline. For each dilution 10 tubes of brain medium were inoculated with 1 ml each. After inoculation the tubes were heated 20 min at 80°C in a hot water bath. The tubes were incubated at 37°C. The tubes were incubated until the number of positive tubes remained constant for 48 hr. The count was estimated on the number of positive tubes.

RESULTS AND DISCUSSION

Counts obtained over a period of 20 years on one lot of spore suspension kept under refrigeration were as follows:

Date	Spores per ml of filtrate
June 21, 1943	8,000,000
June 21, 1943	7,000,000
July 6, 1943	8,000,000
July 28, 1943	6,000,000
August 4, 1943	7,000,000
August 19, 1943	9,000,000
October 30, 1943	8,000,000
December 21, 1943	7,000,000
December 21, 1943	8,000,000
July 16, 1945	30,000,000
August 3, 1945	800,000
January 2, 1952	4,000,000
April 24, 1963	30,000,000

These data show that under the conditions of this study there has been very little change in the spore count over 20 years. Two exceptions are noted. Because the spores were produced in a media containing solid tissue and because it is very difficult to separate the cells from the fine suspended solids, a mass or clump of the spores could have been transferred to the saline. Thermal resistance studies on the spores in 1943 showed the spores had a thermal resistance of more than F_0 9 $z = 18$ but were destroyed at F_0 11 $z = 18$ (Vinton *et al.*, 1946).

Spores undergo changes during storage. Some sporeformers may germinate in the same media in which they are formed. Thus we have a culture in which cells of all stages of growth are present, vegetative cells, cells in the process of forming spores and free spores. Microscopic examination of the filtrate at various times showed this was the condition of this filtrate.

The number of spores in the filtrate have remained basically constant for 20 years at 40°F, and studies were initiated to determine whether the thermal resistance of these spores remained constant.

To determine this in April 1963 tubes were prepared from the original spore suspension, sealed and processed in an oil bath at 238.1°F $z = 18$ (Gross *et al.*, 1946). The spores survived F_0 3 but were destroyed at F_0 5. To see if the spores would germinate and reproduce in the spent medium, 12 ml of the original spore suspension were transferred to an 80°F incubator and incubated for 14 days. There was no indication of growth during the incubation period. After 14 days tubes were prepared and thermal resistance checks were made. The spores showed a thermal resistance of F_0 3 with destruction at F_0 5.

A few of the original spores were transferred to fresh dextrose brain broth media and incubated 6 days at 37°C. Thermal resistance checks on this new suspension showed a resistance of F_0 9 $z = 18$ with destruction at F_0 11 $z = 18$.

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The Influence of Chemical Additives on the Heat Resistance of Salmonella typhimurium in Liquid Whole Egg

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(Manuscript received May 27, 1963)

SUMMARY

The heat resistance of *Salmonella typhimurium* in liquid whole egg at pH 5.5 and 55°C was reduced significantly by a number of chemical additives. Of these, the most effective were β-propiolactone, ethylene oxide, and butadiene dioxide. Additional advantages of ethylene oxide and β-propiolactone were the elimination of *S. typhimurium* from heavily inoculated whole-egg slurry during storage at 0°C and a markedly increased lag phase of growth of the test organism in egg incubated at 30°C. Butadiene dioxide, although not tested at 0°C, was most effective in reducing heat resistance at pH 5.5 and 55°C, and also greatly increased the lag phase of the test organism at 30°C. Unfortunately, this latter compound is carcinogenic to mice.

Acetic or lactic acid, used to lower the pH of liquid whole eggs, decreased the heat resistance of *S. typhimurium* more effectively than did hydrochloric acid, especially at pH 5.5 and 6.0. Formic and propionic acids also were more effective than HCl when all were tested at pH 5.5. Differences in degree of dissociation between hydrochloric and the organic acids are thought to account, in large measure, for these observations.

The occurrence of *Salmonella* species in liquid whole egg as well as in dried egg is well known, and a number of cases of salmonellosis have been traced to such contaminated egg in bakery products (Tanner and Tanner, 1953; Dack, 1956; Taylor, 1960).

The possibility of reducing the heat resistance of bacterial spores by chemical additives has been investigated for a number of years (Andersen and Michener, 1950). Encouraging results in altering the heat resistance of bacterial spores by such additives have recently been reported by Andersen and Michener *et al.* (1950), Denny and Bohrer (1959), and Michener *et al.* (1959). The pasteurization of liquid egg is, at best, a borderline operation since the time-temperature relationship cannot be increased, because of the coagulation of the albumen. Therefore, an attempt was made to decrease the heat resistance of *Salmonella typhimurium* in liquid egg by the addition of various chemicals. No consideration was given to their ultimate effect on drying or to the chemical, physical and sensory properties of the dried product. These factors are relevant objectives for future investigations. The results concerning chemical additives are described herein.

MATERIALS AND METHODS

Test organism. *S. typhimurium* was used in all experiments since it usually occurs in eggs at a higher frequency than other *Salmonella* species (Taylor, 1960). The culture was obtained from Dr. Walter W. Sadler, School of Veterinary Medicine, University of California, Davis.

Media. Trypticase soy broth (Baltimore Bacteriological Laboratories; BBL) plus 1.5% agar (Difco) was used for the preparation of cultures for cell suspensions as well as the plating medium, because BBL soy agar was not in stock when the experiments were started. Bismuth sulfite agar (Difco) was used as the confirmatory medium. These were prepared in the usual manner. Fresh media were used for each experiment.

Cell suspension. An actively growing culture was used to inoculate trypticase soy agar slants. After 24 hr at 37°C the growth was washed off

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into sterile 0.9% saline solution. The cell suspension was stored at 4°C and replaced every five weeks although the heat resistance of any single suspension remained constant for 7–8 weeks. Before use, the suspension was filtered through sterile Whatman No. 1 filter paper to remove clumps and adjusted to contain 2×10^{7} cells/ml.

Preparation of whole egg. Eggs were broken into a sterile Waring blender and homogenized. Their pH was adjusted to 5.5 with sterile 1*N* HCl and 1*N* organic acids or 1*N* NaOH to 7.5. They were strained through sterile cheesecloth, and then pasteurized 8–10 min at 60°C. For growth-curve experiments, eggs were dipped in alcohol, flamed, and broken into a sterile container. The usual aseptic precautions were taken before pasteurization. Sterility tests were always conducted by adding 1 ml pasteurized egg to 9 ml trypticase soy broth and incubating 24 hr at 37°C.

Cell destruction tests. The rate of cell destruction was determined in a three-neck 1,000-ml Woulff flask. It was fitted with a glass stirrer inserted through the center hole. One-hundred-ml samples of pasteurized egg containing the test chemical in the desired concentration (w/v) were heated with continuous stirring for 10 min in this flask in a water bath at the desired temperature (±0.1°C). One ml of cell suspension was then introduced to give an initial concentration of 2×10^{5} cells/ml of egg slurry. One ml of heated egg was withdrawn at regular intervals and immediately diluted to 1×10^{-3} of the original concentration. Then serial-dilution plate counts were made in duplicate. Sampling, based on exploratory tests, was continued until 90–99% of the bacteria were killed. The plates were incubated 48 hr at 37°C before colonies were counted. The advantage of the above method is that the test organism remains in contact with high concentrations of chemicals only during the actual heating period. Used for studies with the low-boiling ethylene and propylene oxides, however, was a technique (involving thermal-death-time tubes) described by Anellis *et al.* (1954). In this latter experiment the two epoxides were chilled and added to chilled whole egg slurry in the cold (approx. 3°C) to prevent their volatilization.

Also tested was the inhibitory effect of the chemicals in inoculated egg slurry held at 0 and 30°C without pasteurization. In addition, sonic oscillation was investigated as a means of destroying *S. typhimurium* in egg slurry. A Raytheon sonic oscillator was used at maximum output (10 kc) in air at 0°C. The slurry was inoculated (2×10^{5} cells/ml), chilled to approximately 0°C, and subjected to sonication. Samples were taken at regular intervals. Numbers of surviving bacteria were determined in the manner described above.

RESULTS

A variety of chemicals did reduce the heat resistance of *S. typhimurium* in liquid whole egg. Fig. 1 shows composite thermal destruction curves for *S. typhimurium* in liquid whole egg adjusted to pH 5.5 with 1*N* HCl. The results are plotted to show the percentage of survivors in relation to time. The destruction caused by heating was not exponential over the whole range. The same type of convex heat-destruction curve was obtained both with and without added chemicals and at the various temperatures and pH values used (see figures and table). It may be that the components of the egg slurry provided protection to the cells, for the most common causes of heat-destruction curves of this type are cells in clumps or in chains. The cells of the culture of *S. typhimurium* commonly occurred singly or in pairs. Clumps of cells were presumably removed by filtration. In contrast, sonication destroyed the bacteria at an exponential rate. This indicated that cells in chains or clumps were not a factor in explanation of the nonlinear heat curves noted above.

A linear relationship was obtained, however, if the logarithms of the *D* values (time in minutes

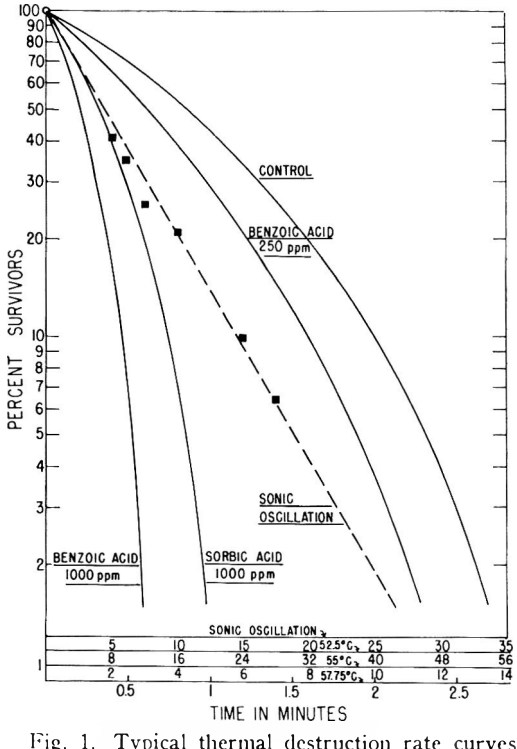

Fig. 1. Typical thermal destruction rate curves for *S. typhimurium* in liquid egg at pH 5.5.

required to destroy 90% of the cells of the test organism) collected in different experiments were plotted against temperature. This relationship is shown in Fig. 2. The addition of sorbic acid or benzoic acid decreased the D values for *S. typhimurium* the same amount at all temperatures tested. β-propiolactone, however, was much more effective in reducing the D values at the lower temperatures, as can be seen by comparing the slopes of the curves for β-propiolactone with those of the control and the other additives tested at this time.

As expected, when the concentrations of the various additives were increased, reductions in D values were greater. However, as can be seen in Fig. 3, no linear relationship existed between concentration and percent reduction in D value. The chemicals generally were relatively more effective at the lower concentrations tested.

The results obtained with most of the additives tested for their ability to reduce the heat resistance of *S. typhimurium* in liquid whole egg at 55°C are summarized in Table 1. From these observations, as well as from those described above, it can be concluded that the heat resistance of *S. typhimurium* in whole egg slurry may be reduced by a variety of different chemicals if the pH of the liquid is adjusted to 5.5. Some of these, generally recognized as safe under the regulations of the U. S. Food and Drug Administration, should

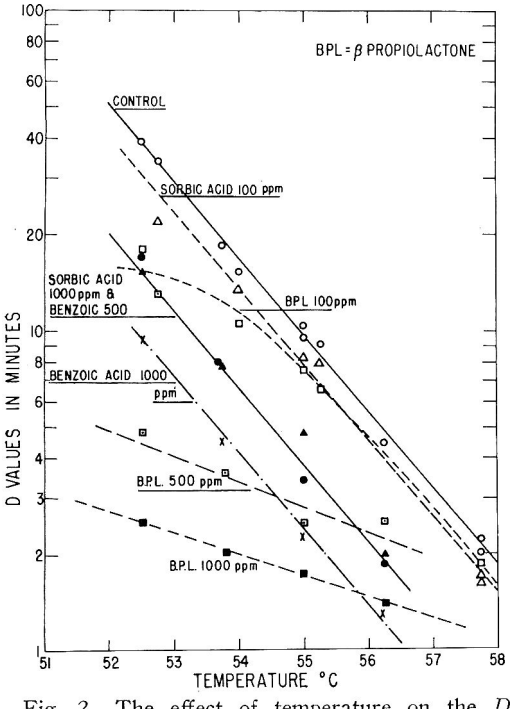

Fig. 2. The effect of temperature on the D value of *S. typhimurium* in liquid egg at pH 5.5.

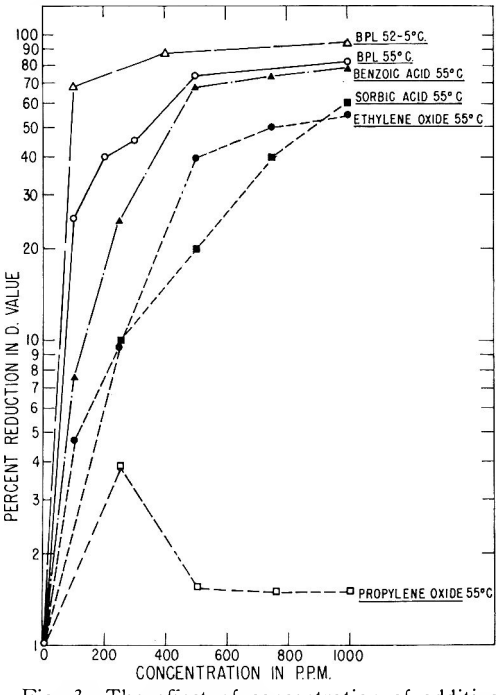

Fig. 3. The effect of concentration of additives on the percentage reduction in D value of *S. typhimurium* in liquid egg at pH 5.5. BPL = β-propiolactone.

be investigated in more detail, especially on a pilot scale simulating commercial conditions. In view of the observations of Lerche (1957) and Lerche and Linke (1958) sensory evaluations should be made as well as volume and solubility measurements on the final product.

Use of organic acids. If required in production specifications, the pH of eggs can be adjusted to 5.5 with hydrochloric acid before pasteurization and spray drying (U. S. Army Quartermaster Corps, 1949; Anellis *et al.*, 1954). Osborne *et al.* (1954) reported that the addition of lactic acid decreased the heat resistance of salmonellae in liquid whole egg as compared with the addition of an amount of hydrochloric acid that would give the same pH. This intriguing observation prompted a study of the effectiveness of acetic and lactic acids as compared with hydrochloric for decreasing the heat sensitivity of *S. typhimurium* in whole egg slurry adjusted to different pH values.

It was found that the effect of hydrochloric acid on the heat sensitivity of *S. typhimurium* was similar to that reported by Anellis *et al.* (1954) for *S. senftenberg*. However, as shown in Fig. 4, 1*N* acetic or lactic acid reduced the heat resistance of *S. typhimurium* more effectively in the liquid whole egg, especially at pH 5.5. Lactic acid was the most effective of the three, causing ap-

Table 1. List of chemicals tested for their ability to reduce the heat resistance of *S. typhimurium* in whole egg at pH 5.5.[a]

Chemical	Temp. (°C)	Concentration (ppm)	% reduction in *D* value
Ascorbic acid	55	1000	+ 0.94
Butadiene dioxide	55	1000	79.5
Butadiene dioxide pH 7.5	55	1000	72.8
Chlortetracyline	57.75	10	0
Chlortetracyline	52.75	30	8.8
Chlortetracyline	55.25	30	7.7
Chlortetracyline	57.75	30	15.0
Chlortetracyline	57.75	30	16.6
Chlortetracyline	57.75	30	0
Dehydroacetic acid	55	2000	71
Formic acid	55		49.2
p-Hydroxybenzoic acid	55	1000	60.4
Lactic acid	55		58.6
Methyl *p*-hydroxybenzoate	55	1000	25.3
Methyl *p*-hydroxybenzoate	55	1000	33
Methyl *p*-hydroxybenzoate pH 7.5	55	1000	25.0
Nisin	55	1000	46.2
Oxytetracyline HCl	52.75	30	8.8
Oxytetracyline HCl	55.25	30	3.3
Oxytetracyline HCl	57.75	30	0
			27.0
Potassium tartrate neutral	55	1000	+18.8
Propionic acid	55		58.75
Propyl *p*-hydroxybenzoate	55	1000	13.2
	55	1000	0
Propyl *p*-hydroxybenzoate pH 7.5	55	1000	36.4
	55	1000	37.8
Sodium acetate	55	1000	+ 1.9
Sodium benzoate	55	1000	52.8
Streptomycin sulfate	52.75	30	16.2
Streptomycin sulfate	55.25	30	3.3
Streptomycin sulfate	57.75	30	12.5
Streptomycin sulfate	57.75	30	16.6
Streptomycin sulfate	57.75	30	7.2
Subtilin	55	30	13.2
Tylosin lactate	55	30	13.0
Tylosin lactate	55	1000	27.6

[a] As indicated, certain chemicals were also tested at pH 7.5. It is to be stressed that a number of chemicals investigated are not on the list of chemicals generally recognized as safe by the Food and Drug Administration of the U. S.

proximately 65% reduction in the *D* value at pH 5.5 and 55°C. Further study, using formic and propionic acids to control the pH to 5.5, confirmed that these two acids also were more effective in decreasing the heat resistance of *S. typhimurium* at 55°C than the addition of the amount of hydrochloric acid needed to reduce the pH of the liquid whole egg to 5.5.

The effect of additives at 0°C. Several additives found to reduce the heat resistance of *S. typhimurium* in liquid whole egg were investigated to determine their effects on the survival of the test organism in the unheated slurry during prolonged storage at 0°C. β-propiolactone was particularly effective in reducing the numbers of viable bacteria in the heavily inoculated egg slurry as shown in Fig. 5. The results with β-propiolactone supplement those already reported by Bruch and Koesterer (1962). It is also interesting that ethylene oxide is more effective than propylene oxide or methyl-*p*-hydroxybenzoate under the conditions of this experiment.

The effect of additives at 30°C. Also determined was the effect of additives on the length of the lag phase of *Salmonella typhimurium* in liquid whole egg (pH 7.5) held at 30°C. Three-week-old cells

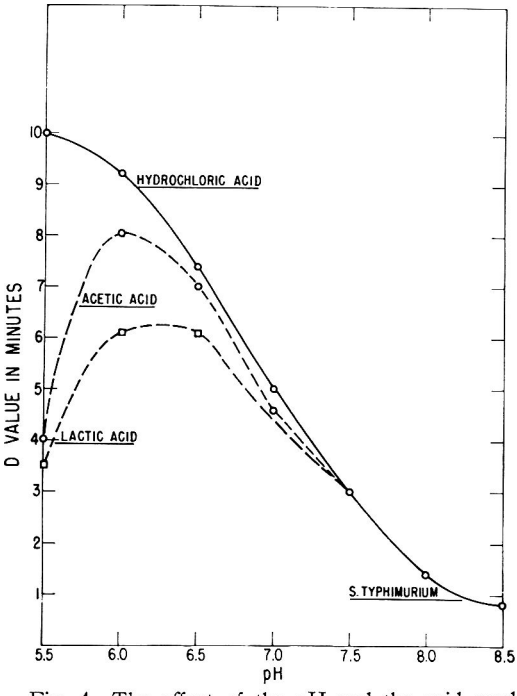

Fig. 4. The effect of the pH and the acid used on the heat resistance of *S. typhimurium* in liquid egg at 55°C.

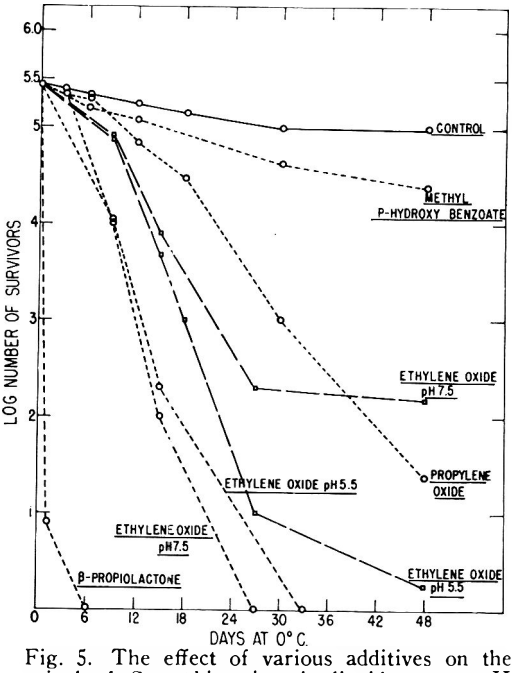

Fig. 5. The effect of various additives on the survival of *S. typhimurium* in liquid egg at pH 7.5 and 0°C. ○ 1,000 ppm; □ 600 ppm.

were used as the inoculum. The initial cell concentration was the same in all cases.

It was found (Fig. 6) that the slopes of the logarithmic phases of growth of the test organism differed markedly in the presence of some additives under the conditions of this experiment. Also, there were considerable differences in maximum cell concentrations developed in 48 hr. The length of the lag phase and the initial decrease in viable cells were also quite variable. It is especially interesting that there is markedly less recovery with the tetracyclines than that observed with some of the other tested additives that have been approved for use as food preservatives in the United States, in concentrations not exceeding 0.1%. However, an explanation is not possible for this observation without further study.

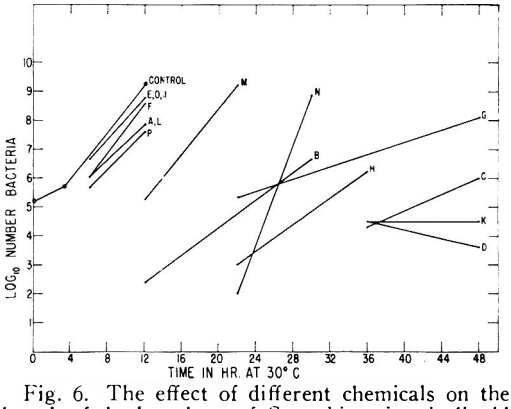

Fig. 6. The effect of different chemicals on the length of the lag phase of *S. typhimurium* in liquid egg at pH 7.5 (only logarithmic growth phases are shown).

A) Benzoic acid	1,000 ppm
B) Butadienedioxide	100 ppm
C) Chlortetracycline	10 ppm
D) Chlortetracycline	20 ppm
E) Dehydroacetic acid	1,000 ppm
F) Ethylene oxide	600 ppm
G) Ethylene oxide	1,000 ppm
H) Ethylene oxide	2,000 ppm
I) P-hydroxy benzoic acid	1,000 ppm
J) Methyl *p*-hydroxybenzoate	1,000 ppm
K) Oxytetracycline	10 ppm
L) Propylene oxide	1,000 ppm
M) β-propiolactone	400 ppm
N) β-propiolactone	600 ppm
O) Propyl *p*-hydroxybenzoate	1,000 ppm
P) Sorbic acid	1,000 ppm

DISCUSSION

It has been well established that acidification of liquid whole egg to pH 5.5 with hydrochloric acid increases the heat sensitivity of salmonellae. Work reported by Osborne *et al.* (1954) with lactic acid, as well as the observations recorded herein, indicate that some of the organic acids increase the heat sensitivity of species of *Salmonella* over that exerted by the amount of hydrochloric acid required to decrease

the liquid whole egg to the same pH value. Although it is well known that organic acids frequently exert specific inhibitory effects on the growth of bacteria, it is also interesting to speculate why some of these increase heat sensitivity of *S. typhimurium* in whole egg slurry to a greater or lesser degree. At least three factors might be involved: $[H^+]$ activity, total concentration of acid, and dissociation. It is thought, however, that $[H^+]$ activity is not a factor, for the pH was 5.5 (within experimental error) in all cases. Concentration may be a factor to a certain degree, but it is to be remembered that the least effective acids (benzoic, *p*-hydroxybenzoic, and sorbic) were used at the highest concentrations (1000 ppm). It seems more likely that the differences in degree of dissociation of the different acids is one major contributing factor that can account for the marked difference noted between hydrochloric and formic, acetic, lactic, and propionic acids. Hydrochloric acid is very highly dissociated at pH 5.5, in comparison to the organic acids under consideration. Since Jacobs (1940) has shown that undissociated organic acids are permeable to the cell, it is thought that this explanation is valid to account for the major differences noted here.

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Heat-Activation Kinetics of Endospores of *Bacillus subtilis*[a]

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SUMMARY

Bacillus subtilis **strain 5230 endospores suspended in water at a concentration of *ca.* 1×10^8 spores per ml were heat-activated at eight temperatures ranging from 5 to 94°C. The response was measured by plate count and recorded as the heat-activated decimal fraction of the total viable count. The method for determining total viable count included a medium containing $CaCl_2$ and Na_2 dipicolinate. This medium enumerated total viable populations, which were equivalent to total direct microscopic counts, without the usual heat-treatment requirement. A method was developed to evaluate the contribution by the plate-count incubation to the total heat treatment. The exposure times were corrected to compensate for the incubation heat treatment. The heat-activation response was obtained throughout the entire range of test temperatures; however, the exposure time required to achieve the response was extended as temperature was decreased. The logarithm of the rate of heat activation was related to the test temperature, i.e., at a lower temperature, a longer time was required for the response. The thermodynamic properties for the system were: $\Delta H^{\ddagger} = 27.9$ kcal; $\Delta F^{\ddagger} = 25.1$–$26.4$ kcal; $\Delta S^{\ddagger} = 4.6$–$8.1$ cal/deg.**

INTRODUCTION

Heat activation has been demonstrated in endospore preparations from many but not all strains of sporeforming bacteria. The substantial but sublethal heat treatment appears to render the endospores less dormant, and in turn enhances the germination process. Consequently, heat activation is often a prerequisite for the enumeration of maximum populations of endospores. Since the early investigations of heat activation (Evans and Curran, 1943; Curran and Evans, 1945, 1946, 1947), a relationship has been evident between the period of time and the temperature level of the heat treatment used to produce this activation, in that lower temperature treatments require extended periods to accomplish the activation resulting from higher temperatures and short times.

Heat shock as a prerequisite to the study of endospore germination has often been used indiscriminately by workers who have not given due consideration to the effect of this heat treatment. However, other workers have advanced beyond a single time-temperature heat shock and have evaluated the influence of the duration or temperature level of the heat activation. Spontaneous germination (Powell and Hunter, 1955), glucose oxidation (Church and Halvorson, 1957), L-alanine germination (Hyatt and Levinson, 1961; Rode and Foster, 1961), and total colony count are some of the systems that have been employed to evaluate heat activation. In each case, increased response corresponded with increased time or temperature of the heat shock.

A number of workers (Desrosier and Heiligman, 1956; Hermier, 1958; Murrell, 1961; Finley and Fields, 1962; Fields, 1963) have systematically evaluated the time-temperature relationships of heat activation as measured by colony count. Although data

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were often reported for several exposure times at each test temperature, the sole reference point for evaluation was usually the one time at each temperature which resulted in maximum germination and growth. These investigations have been limited also by the narrow range of temperatures used, by the possibility of thermal inactivation occurring simultaneously with the heat activation at the high temperatures employed, and by the occurrence of heat-induced dormancy. These early investigations did not characterize the total spore suspension, because in no instance did they evaluate the total viable populations.

The requirement for heat activation may be reduced by limited storage at refrigeration temperature (Powell and Hunter, 1955; Halmann and Keynan, 1962) or may be removed completely (Church and Halvorson, 1956; Halvorson *et al.*, 1961). Ball (1961) recently suggested a relationship between aging and heat activation by asking whether there must be heat activation or just activation at any temperature.

This investigation was initiated to further characterize the heat-activation properties of bacterial spores. The classical definition of heat activation, that of rendering the endospores less dormant and thus allowing the production of maximum colony formation, is used throughout this report. It has the following four objectives:

1) To introduce a medium that permitted the enumeration of total viable populations of spores without the use of heat shock.

2) To describe a method for evaluating the contribution of plate count incubation to the total heat-activation treatment.

3) To present data on the kinetics of heat activation through a range of temperatures from 5 to 94°C.

4) To report the thermodynamic properties of the system.

MATERIALS AND METHODS

Test organism. *B. subtilis* strain 5230 obtained from C. F. Schmidt (Continental Can Co., Chicago) was used throughout the study. Its identity as *B. subtilis* was confirmed in our laboratory according to the methods of Smith *et al.* (1952). Curran and Evans (1945) originally reported on the heat activation requirements of spores of this strain which they coded as 15 μ. Stock cultures were maintained at 5°C.

Media. Tryptone-glucose-extract agar (Difco), fortified with minerals (excluding K_2HPO_4) designated as G_b minerals by Pelcher *et al.* (1963), was used as a sporulation medium, as a recovery medium, and as a stock culture medium. Growth on fortified tryptone-glucose-extract agar (fortified TGE), in contrast to several other standard media, resulted in spores which were comparatively free of sporangia, thus eliminating the requirements of a lysozyme treatment for the removal of sporangial fragments. Also, fortified TGE, when compared with several standard recovery media, yielded the greatest enumeration of spore suspensions by plate count, thus lessening the possibility of nutritional or germinal deficiencies.

A special medium which contained germination agents, $CaCl_2$, and sodium dipicolinate (Na_2DPA), was used to enumerate total viable populations. This medium was termed CaDPA-TGE and was fabricated in the following manner: concentrated, fortified TGE, formulated to contain 90% of the normal water concentration, was treated in the conventional manner through the tempering stage prior to use in the plating procedures; a prescribed amount of sterile 2*M* $CaCl_2$ solution was added to the tempered, concentrated, fortified TGE and was thoroughly distributed by mixing; a prescribed amount of sterile 0.5*M* Na_2DPA (prepared from 2,6-pyridinedicarboxylic acid; mol wt 197.14; K & K Lab., Jamaica, N. Y.) solution was added to the preceding mixture; after the CaDPA-TGE was mixed, it was dispensed immediately. The concentrations of $CaCl_2$ and Na_2DPA in the resultant medium respectively were 50 m*M* and 40 m*M* and were consistent with those reported to induce germination (Riemann and Ordal, 1961).

Preparation of spore suspension. The inoculum, an 18-hr shake culture in 2% peptone broth grown at 37°C, was dispensed in 0.5-ml quantities into Petri plates (150×25 mm) containing 75 ml of fortified TGE and distributed over the entire surface of the medium. After 48-hr incubation at 37°C, the growth was harvested in cold sterile distilled water. The subsequent spore suspension was washed and freed of debris and vegetative cells in a manner similar to the method of Long and Williams (1958). The spore material was separated by centrifugation for 20 min (Servall SS3, GSA 5.75 head) at 3000 rpm (1465×G) for the initial separation and, for each subsequent separation, at a speed of 500 rpm greater than the previous time up to a maximum of 8000 rpm (10400×G). After removal of the debris layer, the bottles containing the remaining spore material and an added 50 ml of washing solution were placed on a rotary shaker (Eberbach, 100

rpm) in the cold room. After 6 hr of shaking, the spore material was completely dispersed. A total of ten washings with cold sterile distilled water resulted in a spore suspension which appeared to be free of foreign material when examined under dark-phase contrast optics (1250×). Throughout the washing procedure, the suspension was kept at *ca.* 5°C. The final spore suspension was stored at −20°C in the lyophilized state until used.

Method for determination of heat activation. At temperatures above 50°C the apparatus described by El-Bisi and Ordal (1956) was used for the application of the heat treatment. The apparatus was simulated at temperatures below 50°C by using 250-ml round bottles placed in appropriate incubators. A magnetic stirrer was used for constant agitation in all cases. In all experiments, a concentration of spores of *ca.* 1×10^9/ml was suspended in sterile, deionized, distilled water. The pH of the several suspensions ranged from pH 6.5 to pH 7.0.

The amount of heat activation is reported as a decimal fraction, i.e., the number of heat-activated spores divided by the total viable number of spores. Using standard plate-count methods, 13 plates were prepared for each sample. The counts from the ten plates poured with fortified TGE enumerated the heat-activated spores, and the counts from the three plates poured with CaDPA-TGE enumerated the total viable population. All plates were incubated 24 hr at 37°C. All results are averages of at least two experiments.

RESULTS AND DISCUSSION

Determination of total viable count. Bacterial spores have been germinated with the addition of $CaCl_2$ and Na_2DPA to the spore suspension (Riemann, 1961; Riemann and Ordal, 1961). This germination occurred without the use of heat shock. Keynan and Halvorson (1962) and Keynan *et al.* (1962) stated that the rate of germination in the presence of Ca and DPA is unaffected by heat shock. In preliminary investigations, spores of *B. subtilis* 5230 germinated in the presence of $CaCl_2$ and Na_2DPA. This observation led to the development of CaDPA-TGE medium, which would bypass the requirement for heat activation and would yield plate counts essentially equal to the total spore population as estimated by direct microscopic count (determined with Petroff-Hausser counting chambers and dark-phase contrast optics). In about 20 separate trials on unheated samples, use of the CaDPA-TGE medium resulted in enumerations of total viable counts which were equal (±5%) to the direct microscopic counts. The 10% variation was within the limits of experimental error in the counting methods. When the CaDPA-TGE was used in the upper range of temperatures of the heat-activation studies, only the viable spores were enumerated and the concurrent thermal inactivation was observed in the results of the total viable counts. By removing the heat-activation requirement of the spores, the CaDPA-TGE medium proved to be a valuable tool in subsequent studies for enumeration of the total viable population of the spore suspension.

Correction of exposure times for heat activation during plate incubation. Plate-count enumeration has specifications for the time and temperature of incubation. Part of the heat treatment during incubation has been suspected of actually resulting in heat activation. The influence of time and temperature of incubation was evaluated because the measurement of heat activation was based on plate-count results. The data in Fig. 1 indicate that enumeration of the population of a non-heat-treated spore preparation was related directly to the time and temperature of incubation. Data on a heat-treated spore preparation show a similar effect, but at correspondingly higher enumeration levels. The growth of the untreated preparation appeared to be due to heat activation during incubation, as did part of the growth of the heat-treated preparation, because of the time-temperature relationships. The counts obtained with the CaDPA-TGE for the same times and temperatures were essentially equal to the total population as estimated from direct microscopic counts. Therefore, lack of optimum growth temperature or the lack of sufficient time to form visible colonies was ruled out as sole reasons for the lower counts at lower temperatures or shorter times. The results obtained here prompted the proposal of a method for evaluating the contribution of plate-count incubation to the total heat treatment.

The data reported in Fig. 2 were used to construct a heat-activation curve resulting from heating at 75°C. This curve was representative of the shape of curves at all test temperatures; however, the time axis would

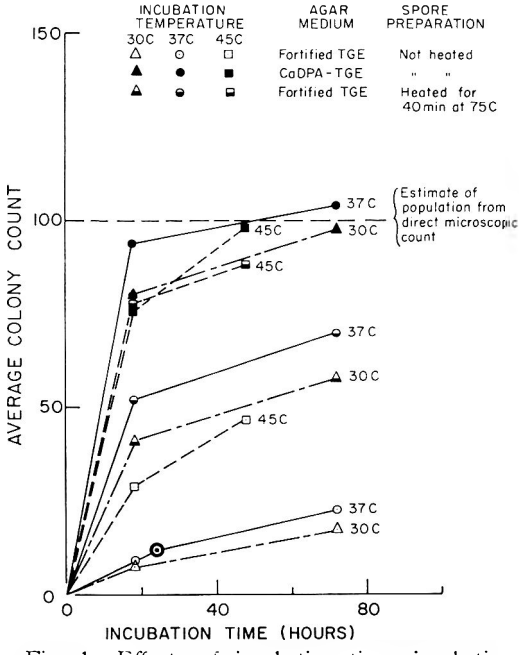

Fig. 1. Effects of incubation time, incubation temperature, and recovery medium on the populations of *Bacillus subtilis* spores enumerated by plate count techniques. The bulls-eye symbol indicates a typical count normally obtained from an untreated spore suspension after 24 hr of incubation at 37°C.

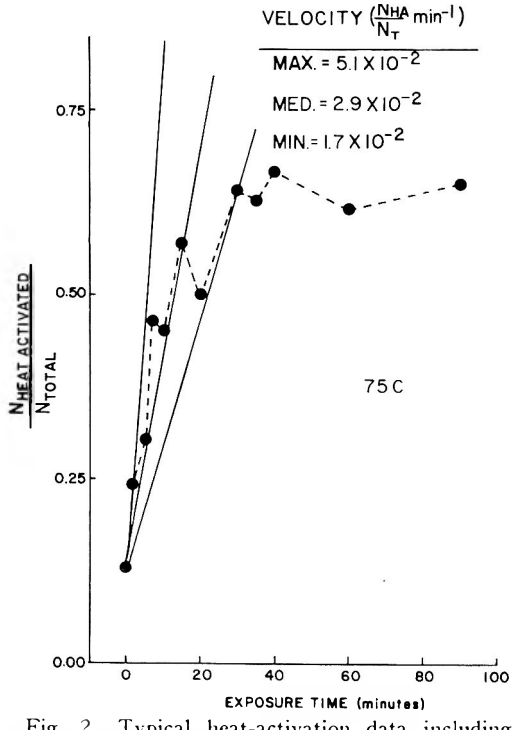

Fig. 2. Typical heat-activation data including velocity specification for *Bacillus subtilis* spores at 75°C.

vary with the temperature. When the number of organisms which have been heat-activated, expressed as the decimal fraction of the total viable population, was plotted vs. the exposure time, in minutes, at 75°C, a nonlinear relationship was observed. Although an infinite number of initial velocities could have been determined, determination was arbitrarily made of three initial velocities of heat activation to include the range of initial velocities and be used as a means of comparing heat activation at several exposure temperatures. The determination of maximum velocity is obvious; minimum velocity was obtained by selecting the period where velocity was greatly reduced and determining the average rate to that point; and medium velocity was determined by the method of visual best fit.

The three initial velocities for each specific exposure temperature were characteristic and were used to construct Fig. 3. These data demonstrate the relationship among the initial velocities at a number of test temperatures. Maximum, medium, and minimum velocities were employed in the plot, and the curve relating these velocities

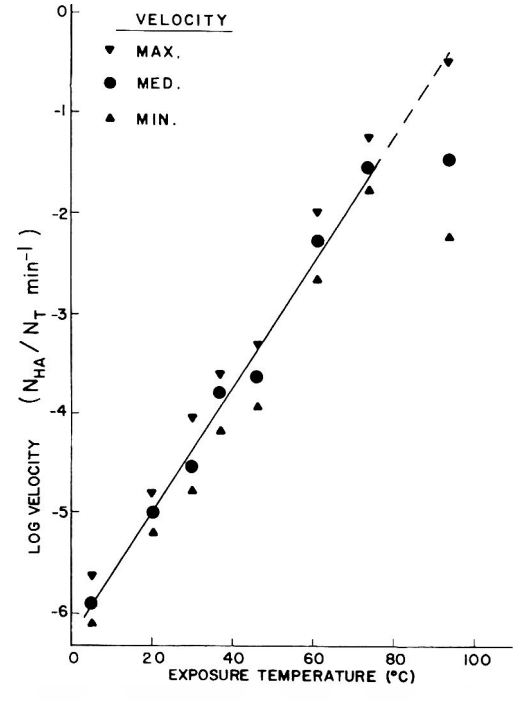

Fig. 3. Relationship of initial velocities of heat activation at eight temperatures.

was positioned by visual best fit. The information in this plot can be used to correct the exposure times for the contribution at 37°C due to plate-count incubation for 24 hr, i.e., the heat treatment received during incubation was evaluated in terms of time at other test temperatures.

The method for the correction of exposure times used to compensate for heat activation during plate incubation involves the following three factors: 1) the ratio of the velocity of heat activation at 37°C to the velocity at test temperature, t°C; 2) the ratio of the amount of heat activation in the fortified TGE to the amount in water for the same period; 3) the time of incubation, 24 hr (1440 min).

The values for the velocity of heat activation ($\frac{N_{HA}}{N_T}$ min^{-1}) at 37°C and at any other test temperature (t°C) were obtained from the curve in Fig. 3. The relationship between these two velocities was expressed as a ratio:

$$\frac{\frac{N_{HA}}{N_T}\ \text{min}^{-1}\ \text{at}\ 37°\text{C}}{\frac{N_{HA}}{N_T}\ \text{min}^{-1}\ \text{at}\ t°\text{C}} \qquad [1]$$

For example, if t°C equals 20°C, Ratio 1 is $(1.3 \times 10^{-4}\ \text{min}^{-1})/(1 \times 10^{-5}\ \text{min}^{-1})$, or 13. Empirically, the heat activation which occurred during plate incubation was considerably lower than that which occurred in water at 37°C for the same period. The lower amount of heat activation may be due to a slower rate of heat activation when the recovery medium (fortified TGE) is the suspending menstruum rather than water; or it may result from insufficient time for visible colony formation by those spores which were heat-activated in the latter period of the 24-hr incubation; or it may be due to a combination of both. Regardless of the reason, the reduction of heat activation in the recovery medium also was expressed as a ratio:

$$\frac{\frac{N_{HA}}{N_T}\ \text{for untreated sample due to plate incubation for 24 hr}}{\frac{N_{HA}}{N_T}\ \text{for sample heat-activated in water for 24 hr (does not include plate incubation)}} \qquad [2]$$

Generally, our value for Ratio 2 was .125/.187, or approximately 2/3.

After the values for Ratios 1 and 2 were resolved, the correction factor was calculated. The amount of time in minutes (the correction factor) to be added to each exposure time at test temperature t°C was determined by multiplying the value for Ratio 1 at t°C, the value for Ratio 2, and the time of incubation at 37°C (1440 min). For example, if t°C equals 20°C, Ratio 1 equals 13, Ratio 2 equals 2/3, and the incubation time equals 1440 min. Thus, the correction factor for 20°C samples was *ca.* 12,500 min. The sum of the correction factor and the exposure time was equal to the corrected exposure time for each sampling time at a specific temperature. Corrected exposure times were used exclusively in the remainder of the study.

It should be noted that the heat activation which occurs during plate incubation affects only those organisms that have not been activated by a previous heat treatment. The use of corrected exposure times, as outlined above, does not compensate for the decrease in numbers of spores not heat-activated by a prior treatment and thus available for activation during plate incubation. However, when the fraction heat-activated in water is low and the fraction available for activation in the agar medium is high, the contribution of the correction factor to total corrected exposure time is considerable and justified. Conversely, as the relationship changes and the fraction available for activation in the agar decreases, the contribution of the correction factor to total time becomes incidental because of extended exposure periods. Therefore, the decrease in precision of the correction factor at extended times is of no consequence.

The contribution of plate incubation was also evaluated in another manner—by deleting the amount of heat activation during incubation from the total measured heat activation. The results obtained in this way appeared similar to those obtained with corrected exposure times; however, as stated previously, the method employing corrected exposure times was used throughout the remainder of the study because it evaluated

total heat treatment and because the graphical representation was more informative.

Kinetics of heat activation. A number of workers have evaluated the time-temperature relationships of heat activation as measured by colony count. Although other data were reported, the sole reference point for evaluation was usually the one time at each temperature which resulted in maximum germination and growth. In a study on heat activation of spore suspensions of *Bacillus globigii*, *Bacillus thermoacidurans*, and PA 3679, Desrosier and Heiligman (1956) observed a relationship between the logarithm of the time at which the maximum response occurred and the temperature of heating, in that as the temperature was reduced the exposure time was increased to yield a similar response. These studies were conducted at temperatures between 55 and 85°C when spores of *B. globigii* were used, and between 65 and 95°C when the other two spore suspensions were employed. Thermal inactivation of the spore suspensions was observed in the latter stages of heating. An investigation of heat activation of *Bacillus coagulans* spores resulted in data from which a Q_{10} value of 5.5 could be calculated for the relationship between time for maximum activation and temperature ranging from 85 to 110°C (Murrell, 1961). Although relationships were stated in these studies, evaluation of the data was limited. Total viable populations during heating were not reported. The contribution of the plate incubation to the total heat treatment was not considered. In addition, the range of test temperatures was comparatively small and the exposure times were conveniently short. These deficiencies were removed in the present study.

Results from heat-activation studies at eight exposure temperatures (5–94°C) are presented in Fig. 4. These data are plotted in a semilogarithmic fashion, which is often used to depict the results of heat activation. The logarithm of the decimal fraction of heat-activated spores is plotted on the ordinate against corrected exposure time on the abscissa. The time axis has been manipulated to illustrate the similarity among heat-activation data at the eight test temperatures. To illustrate the variation in magnitude on the time axis, note that the data for the 94°C heat-activation study cover a period of 90 min whereas the data

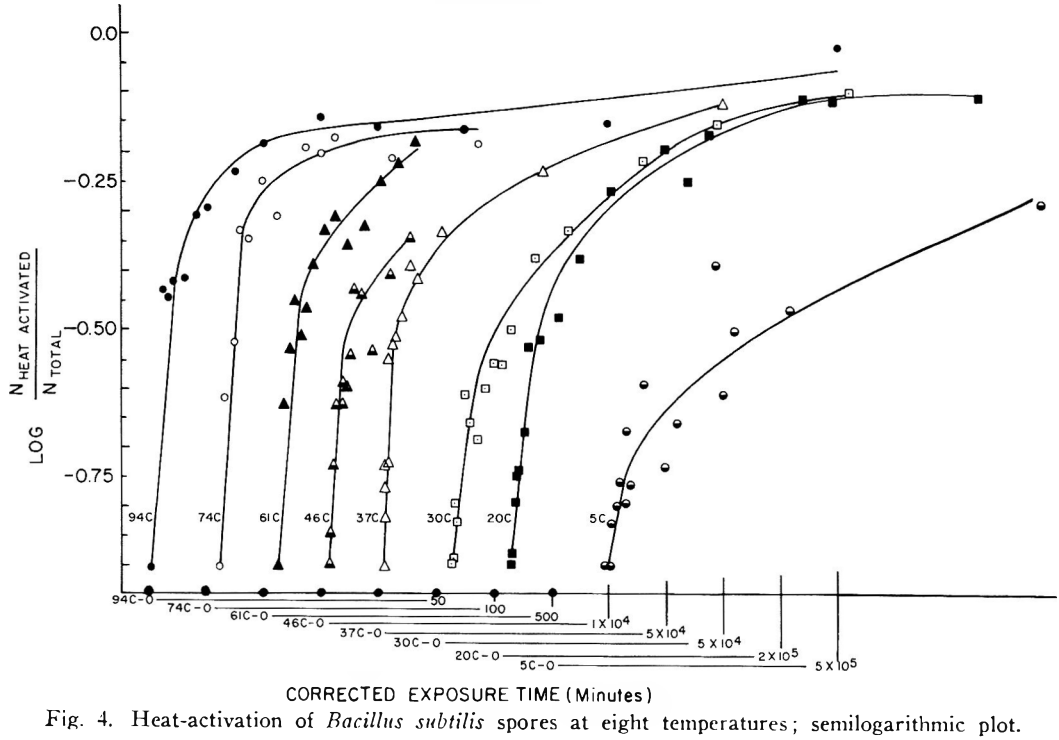

Fig. 4. Heat-activation of *Bacillus subtilis* spores at eight temperatures; semilogarithmic plot.

for the 5°C study encompass a period of 525 days. Within the range of temperatures studied, these data indicate that the heat-activation response obtained at the lower temperatures over extended periods is similar to the response obtained at the higher temperatures during short periods of exposure.

Thermodynamic properties of heat activation. Rates of temperature inactivation of unicellular organisms have been examined by comparison to rates of enzymatic inactivation (Wood, 1956). In kinetic studies, Charm (1958) reported the average energies of inactivation of bacterial spores by heat under several conditions. Recently, whole-cell suspensions were employed to determine the thermodynamic properties of thermal inactivation of *Staphylococcus aureus* (Stiles and Witter, 1962; Stiles, 1963). In order to determine the kinetics of a heat-activation system, initial rates and subsequent rate constants had to be obtained. The data in Fig. 5 were used for these rates. The logarithm of the reciprocal of the decimal fraction of spores remaining to be heat activated was plotted against the corrected exposure time on the manipulated time axis. The designation of the ordinate was predicated on the need for a positive slope (accomplished by the reciprocal), the use of only those spores remaining available to enter into the heat-activation reaction, and the previous observation that the heat-activation response resembled a reaction of the first order. The latter statement was based on data that indicated a specific fraction of the total population of spores was activated per unit time regardless of the initial population level. The reason for the leveling of the curves, especially at the higher temperatures, was not readily evident; however, heterogeneity of the spore suspension could be a possible explanation. Therefore, the initial portions of the curves, prior to the distinct change in slope, were used to determine average-rate constants for subsequent use.

The reaction velocities for the test temperatures were used in the Arrhenius plot shown in Fig. 6, where the logarithm of the reaction velocity is plotted vs. the reciprocal of absolute temperature. The method of the sum of least squares is used for the

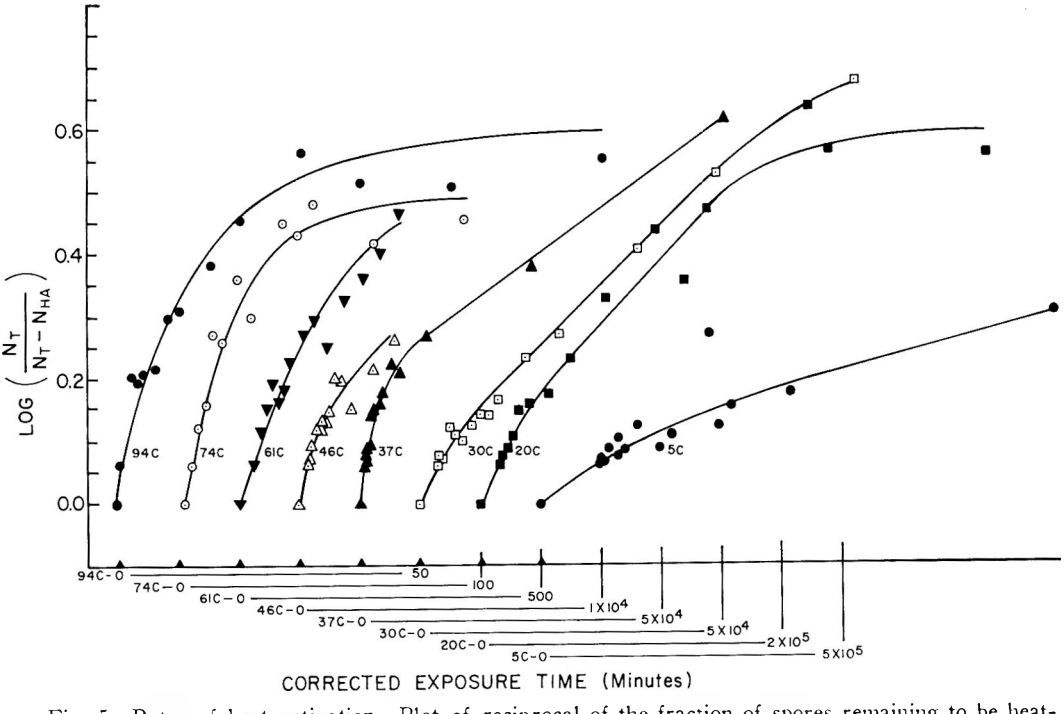

Fig. 5. Rates of heat activation. Plot of reciprocal of the fraction of spores remaining to be heat-activated vs. corrected exposure time.

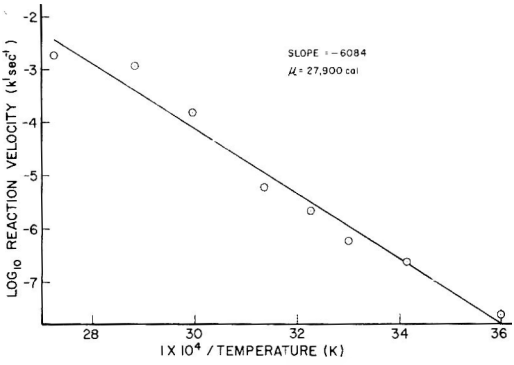

Fig. 6. Effect of temperature on the rate of heat activation. An Arrhenius plot of the reaction velocity vs. the reciprocal of absolute temperature.

fitting of the straight-line curve. A slope of −6084 was obtained, and, accordingly, a μ of 27.9 kcal was determined for the system (Fruton and Simmonds, 1958). These data were used for the calculation of other thermodynamic constants.

When the activated complex is formed from reactant molecules, $\Delta H^{\ddagger}$, $\Delta F^{\ddagger}$, and $\Delta S^{\ddagger}$ respectively represent the standard changes in heat content or enthalphy, free energy, and entropy. Standard calculations and equations were employed to determine these thermodynamic constants as stated by Stearn (1949). The maximum difference (RT) between $\Delta H^{\ddagger}$ and μ for the system was .729 kcal, which was considered insignificant. Therefore, assuming that $\Delta H^{\ddagger}$ is approximately equal to μ for our system, the standard enthalpy ($\Delta H^{\ddagger}$) was 27.9 kcal. The standard free energies ($\Delta F^{\ddagger}$) were calculated from the rate constants (Stearn, 1949). The $\Delta F^{\ddagger}$'s for the 8 temperatures ranged from 25.1 to 26.4, and the average $\Delta F^{\ddagger}$ was 26.0 kcal. Standard entropy ($\Delta S^{\ddagger}$) was determined by difference. The values ranged from 4.6 to 8.1 cal/deg, and the average $\Delta S^{\ddagger}$ was 6.1 cal/deg. Using the basic assumptions and the approach suggested by Stearn (1949), certain additional observations can be obtained from these data. Assuming a value of 20 kcal per strong bond and dividing the $\Delta H^{\ddagger}$ by this value, the indication is that *ca.* 1.4 strong bonds have been broken. Furthermore, assuming a $\Delta S^{\ddagger}$ of 12 cal/deg for each bond, regardless of strength, a theoretically calculated $\Delta S^{\ddagger}$ is estimated at *ca.* 18 cal/deg. This calculated $\Delta S^{\ddagger}$ is greater than, but reasonably near, the experimental value. The possible breaking of several weak bonds instead of a strong bond, or several weak bonds plus a strong bond, is not feasible, because of the subsequent increase in the calculated $\Delta S^{\ddagger}$ and, accordingly, the increased deviation from the experimental value. These values are consistent with the speculation that heat activation is indeed disrupting a system that requires a rather severe treatment for activation.

Kinetic studies measuring over-all systems, as we have employed here, merely give an indication of possible events occurring during heating. Obviously, one cannot prove mechanisms from kinetics alone. The rate-limiting reaction is being measured, but the precise role of this reaction is not readily apparent. One possible explanation of the influence of heat would be the breaking of dormancy by structural reorientation of components of the spore that, in turn, would lead to the loss of the characteristic integrity of the resistant spore. This reorientation may involve one of a number of components of the spore. However, regardless of the specification, these kinetic data should prove to be a useful guide and support for additional study.

These kinetic data appear to indicate little difference between high-temperature heat activation and what has been termed "aging at low temperatures." In fact, it appears that activation is occurring throughout the entire range of test temperatures and is a function of time. Complementary investigations are necessary to correlate the observed similarities throughout the temperature range tested; nevertheless, the data indicate that studies at lower temperatures will accomplish heat activation over a longer period and thus allow heat activation to be slowed for more detailed study.

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Voges-Proskauer-Positive Yeasts Isolated from Frozen Orange Concentrate[a]

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(Manuscript received November 29, 1963)

SUMMARY

Seven Voges-Proskauer-positive yeasts were isolated from 290 cans of frozen concentrated orange juice representative of the 1962 season's pack. Taxonomy studies of 4 isolates showed the presence of 2 strains of *Saccharomyces*. Each of the identified yeast strains grew and produced diacetyl in 12° Brix orange juice and 42° Brix orange concentrate, then subsequently removed it. Reagent-grade diacetyl added to a 42° Brix sucrose solution inoculated with one of the test organisms was reduced 95% after 7 days at room temperature. The metabolic process involved in removing diacetyl from a product by yeast is not known.

One of the principal spoilage hazards in the production of frozen concentrated orange juice has been the development of off-flavors characterized as being similar to "buttermilk." This type of spoilage was first reported by Hayes (1951) and Murdock *et al.* (1951). Those investigators were of the opinion that the bacteria responsible were principally *Bacillus* organisms. In 1954 Byer developed a method for the detection of diacetyl or acetylmethylcarbinol in frozen concentrated orange juice. In the same year Hill *et al.* reported on a colorimetric method for the detection of microbial spoilage in citrus juices. These original citrus workers used a modification of the Voges-Proskauer (VP) reaction for the detection of diacetyl and acetylmethylcarbinol (AMC). This test is highly specific for these compounds in concentrations of less than 1 ppm (Minute Maid). According to Barrett (1936), a color reaction will also be obtained for 2,3-butanediol (2,3-butylene glycol) in concentrations of 40,000 ppm and higher. This test is based on the development of a pink to ruby color after the addition of alpha-naphthol and 40% KOH creatine solutions to a glucose phosphate peptone culture.

Diacetyl has been responsible for undesirable off-flavors in products other than citrus juices and concentrates. Diacetyl, for example, is of concern in the brewing of beer where a concentration of 0.5 ppm is said to give a noticeable off-flavor, particularly in lager beers (Rose, 1963). This compound is known to be produced by certain pediococci which are the causative agents of beer "sarcina" infection and bacterial infection (Burger, 1958b; Rose, 1963; West, 1952). However, recent workers have found diacetyl to be produced in beer by yeast in pure culture (Wood, 1961).

The presence of diacetyl and/or AMC was reported by Fields (1962) to be an important index to the microbial quality of apple juice. Molds and yeasts were found to produce VP reactants in this product. Values above 1.6 ppm were said to indicate poor sanitary conditions.

In the citrus industry, off-flavors due to a build-up of diacetyl have always been attributed to bacteria. The possibility that other organisms such as yeast may be involved has never been investigated. Beisel *et al.* (1954) found that certain strains of yeast were capable of giving a positive VP reaction in orange juice serum. However, no further studies were made.

This paper reports on the production of diacetyl and AMC in orange juice and concentrate by yeast.

PROCEDURE

Source of test organisms. Two hundred and ninety cans of frozen concentrated orange juice representative of the 1962 season's pack were

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pour-plated over a 10-week period. During this interval 100 yeast colonies were picked and streaked on orange serum agar slants. Each culture was inoculated into sterile orange juice, incubated 48–72 hr at 30°C, and then examined microscopically for growth. If growth occurred, they were checked for VP reactants. All yeast grew in orange juice. Of the 100 cultures examined, only 7 gave a positive VP reaction. Of the 7 isolates, 4 were sent to the USDA, Northern Utilization Research and Development Division, Peoria, Illinois, for identification. They were identified by Dr. Lynferd J. Wickerham as *Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae*. They are respectively referred to herein as yeast strains Y-17 and Y-27.

Bacteriological. Two yeast suspensions were prepared, one from each strain of *Saccharomyces*. Sterile 12° Brix orange juice and sterile 42° Brix orange concentrate in Erlenmeyer flasks were inoculated with each yeast strain so that the product, after inoculation, contained approximately 100,000 yeasts per ml. Just prior to each plating, the contents of the flasks, held at room temperature (74–76°F), were agitated with a magnetic stirrer. Orange serum agar was used as the growth medium, and the plates were counted after 72 hr of incubation at 30°C. Immediately after the juice and concentrate were plated, a sample was drawn aseptically for diacetyl and AMC analysis. At this time the product was also analyzed for Brix, acid and B/a ratio (Table 1).

Voges-Proskauer test. The method used was that of Byer (1954). This procedure involved distilling 300 ml of 12° Brix orange juice and collecting 25 ml of distillate in a graduated cylinder. To the distillate was added alpha-naphthol and KOH creatine solutions. One minute after the reagents and distillate portion were mixed, colorimetric readings were made on a Lumetron Model No. 401 with a 530-μ filter. The solution was again read after a 10-min period. In this paper the concentration of diacetyl is that obtained from the 1-min reading, and the 10-min determination minus the first is recorded as ppm of AMC. The ppm of diacetyl was obtained by applying the Lumetron reading in percent light transmission to a calibration curve prepared from samples of water containing known amounts of diacetyl.

According to Byer (1954), the diacetyl color reaction is almost complete after 1 min. For AMC, the color development has just about reached its maximum intensity in 10 min when the concentration is less than 5 ppm (Hill and Wenzel, 1954). For higher values of AMC 30 min are said to be required for maximum color intensity (Byer, 1954). In this paper the 10-min reading is used for the sake of convenience and since most AMC values were less than 5 ppm.

RESULTS AND DISCUSSION

It was noted in sterilizing juice samples for this study that the heated product was apparently higher in diacetyl than the unheated sample. Further investigation showed that heating juice in the autoclave under steam pressure gave higher diacetyl values than it did in flowing steam. This is shown in Table 2, where 4 different samples of 42° Brix orange concentrate were heated 30 min

Table 1. Brix, acid, and B/a ratio of orange juice and orange concentrate during test period.

Days at RT[a]	12° Brix orange juice			42° Brix orange conc.		
	Brix	Acid	Ratio	Brix	Acid	Ratio
Yeast strain Y-17						
0	11.95	0.98	12.20	42.17	3.20	13.18
1	10.71	0.92	11.64	42.01	3.12	13.46
2	5.55	0.96	5.78	39.99	3.30	12.11
3	5.38	0.90	5.97	36.06	3.38	10.66
4	5.64	0.96	5.85	33.80	3.44	9.82
5	5.06	0.86	5.88	NC[b]	NC	NC
7	5.02	0.92	5.45	NC	NC	NC
Yeast strain Y-27						
0	11.91	0.84	14.20	42.15	3.10	13.60
1	10.11	0.98	10.32	40.99	3.12	13.13
2	5.55	1.00	5.55	39.29	3.30	11.90
3	5.60	0.96	5.83	35.26	3.36	10.49
4	5.62	0.92	6.10	32.80	3.42	9.59
5	5.06	0.90	5.62	NC	NC	NC
7	5.16	0.92	5.60	NC	NC	NC

[a] RT = room temperature.
[b] NC = not checked.

Table 2. Voges-Proskauer reactants (ppm) in 42° Brix orange concentrate heated at various temperatures.

	Date packed							
	Jan. 2 1962		Feb. 16 1962		April 12 1963		May 17 1963	
Variable	Diac.	AMC	Diac.	AMC	Diac.	AMC	Diac.	AMC
Control (no heat)	0.12	1.28	0.18	1.62	0.22	1.78	0.18	1.72
30 min flowing steam	0.421	1.28	0.65	1.65	0.69	1.81	0.86	1.84
20 min at 250°F	0.69	1.41	0.69	1.71	1.50	1.80	0.91	1.99

Diac. = Diacetyl; AMC = Acetylmethylcarbinol.

in flowing steam and 20 min in the autoclave at 250°F. It is interesting to note little if any difference between heated and unheated samples in concentration of AMC. Heat apparently causes the conversion of components in the juice to one or more compounds giving positive VP reactions, presumably diacetyl. According to Beisel *et al.* (1954), heated solutions of sucrose, fructose, or glucose at pH 3.5 gave a qualitative VP test. This author, however, was unable to confirm his findings. For example, a 42° Brix solution of sucrose and one of dextrose were acidified with citric acid to pH 3.5, heated at the temperatures indicated in Table 2, and then checked for VP reactants by the distillation procedure. All tests gave negative results.

The growth of the test strains of yeast in 12° Brix orange juice and 42° Brix orange concentrate are shown in Figs. 1 and 2. It will be noted that both yeast strains gave similar results. As the yeast population increased there was a corresponding increase in diacetyl until a maximum of 6.5–7.5 ppm was reached in orange juice, and 7.2–9.1 ppm in 42° Brix orange concentrate. After these levels were obtained, a rapid decrease in diacetyl occurred. The 12° Brix juice samples, for example, contained less diacetyl after 4 days at room temperature than they did at the start of the study. Orange juice and concentrate were examined organoleptically when maximum concentration of diacetyl was obtained. The samples tasted very yeasty, which apparently prevented detection

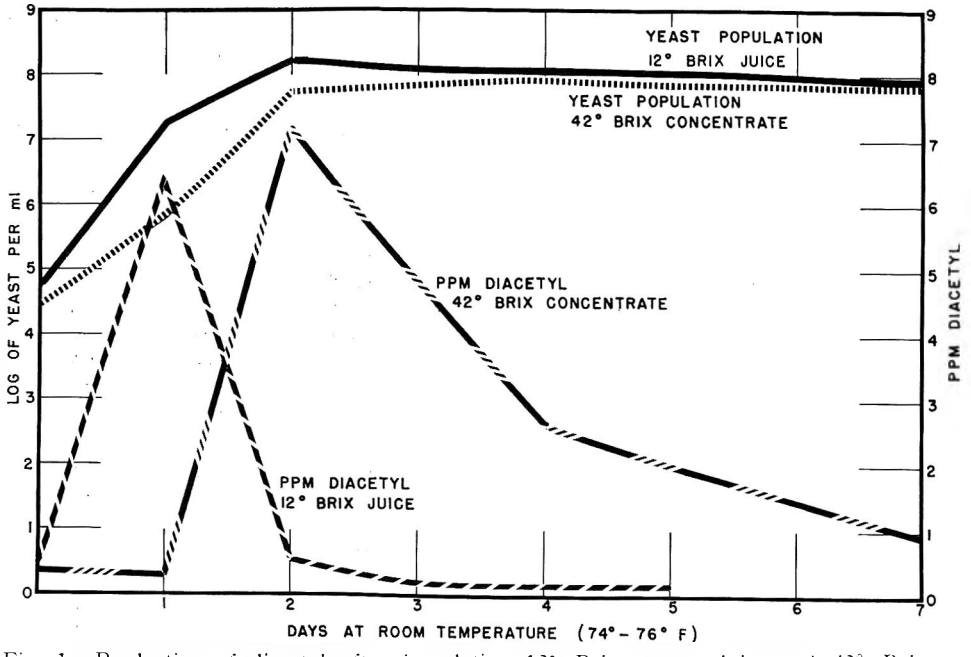

Fig. 1. Production of diacetyl after inoculating 12° Brix orange juice and 42° Brix orange concentrate with yeast strain Y-17.

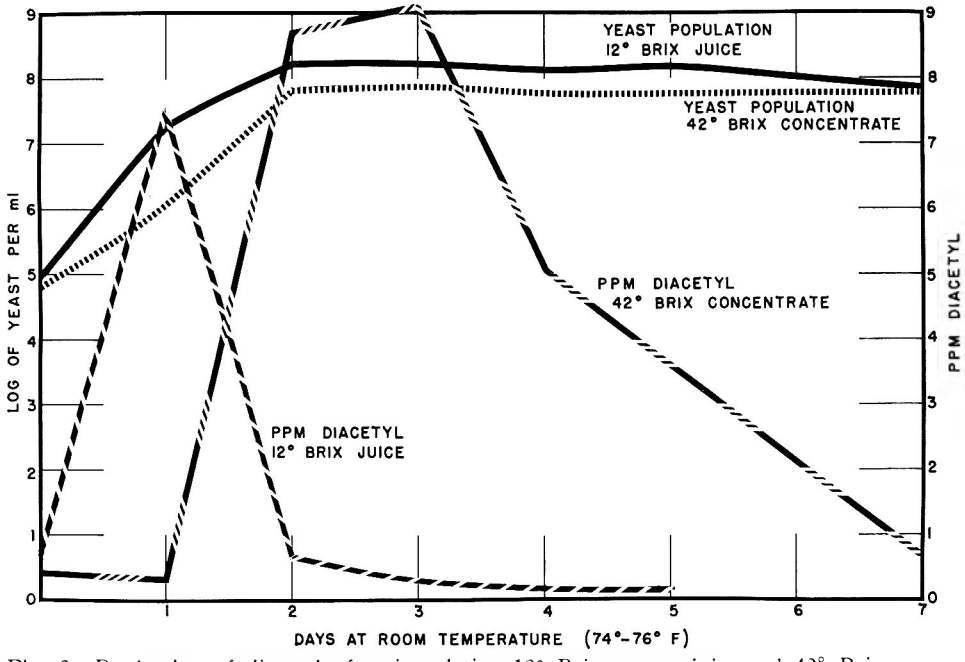

Fig. 2. Production of diacetyl after inoculating 12° Brix orange juice and 42° Brix orange concentrate with yeast strain Y-27.

of the characteristic buttermilk flavor associated with this type of spoilage.

In another experiment, diacetyl was added to a sterile 42° Brix sucrose solution adjusted to pH 3.8 with citric acid and inoculated with yeast strain Y-27 so as to contain approximately 100,000 organisms per ml. The syrup was plated and analyzed for diacetyl in the manner previously described. The data show (Fig. 3) that as the yeast

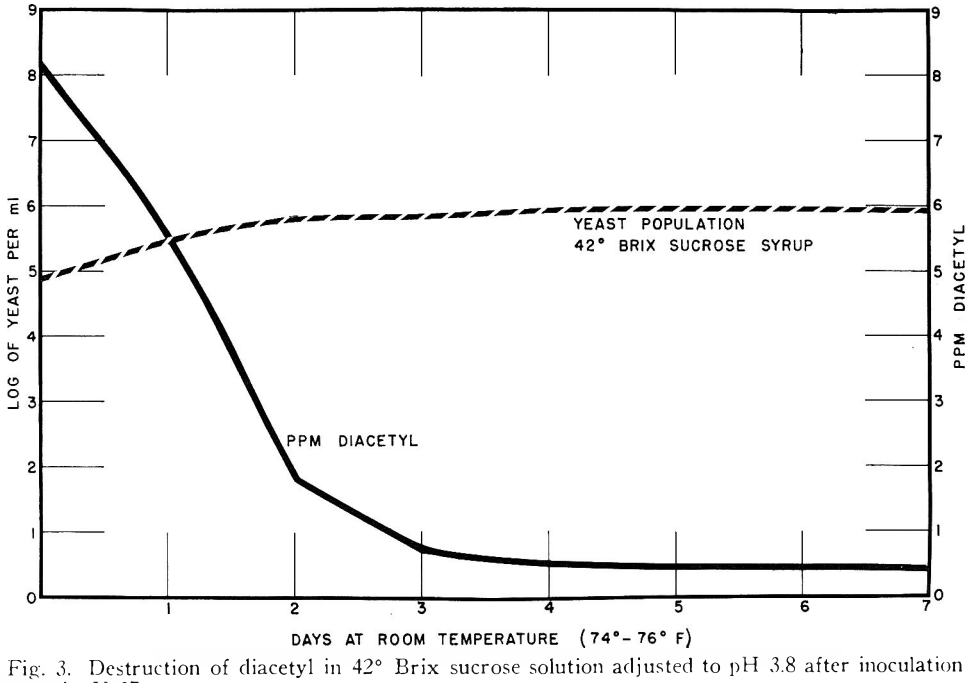

Fig. 3. Destruction of diacetyl in 42° Brix sucrose solution adjusted to pH 3.8 after inoculation with yeast strain Y-27.

Table 3. Concentration of AMC in orange juice, orange concentrate, and sucrose syrup (results expressed as percentage of AMC to total conc. of diac. and AMC in sample).

Days at RT[a]	Yeast strain Y-17		Yeast strain Y-27		
	12° Brix juice	42° Brix conc.	12° Brix juice	42° Brix conc.	42° Brix syrup
0	81	82	77	82	9
1	64	91	61	93	28
2	87	60	86	51	49
3	93	73	87	53	70
4	92	84	92	74	76
5	96	NC[b]	94	NC	NC
7	NC	93	NC	94	81

[a] RT = room temperature.
[b] NC = not checked.

population increased, diacetyl decreased, dropping from 8.2 ppm to 0.38 ppm during the 7-day test period. No change in diacetyl concentration occurred in another sample of uninoculated sucrose syrup, indicating that yeasts are necessary to removal of this compound.

It is apparent that the two strains of yeast under investigation produce diacetyl in citrus juice and concentrate, and subsequently remove it. Burger *et al.* (1958a) in studies on the formation and prevention of diacetyl in beer, also observed this phenomenon. They found that the addition of liquid yeast, at the rate of 0.75 lb per barrel of beer, is sufficient to remove the diacetyl flavor from even a badly contaminated beer. Those investigators were of the opinion that the disappearance of diacetyl in beer is possibly due to an enzyme system in the yeast which actually destroys the diacetyl.

Although it is not known what metabolic processes are involved in the removal of diacetyl from orange juice, it does appear from the data in Table 3 that there might be some conversion to AMC, especially after the third day of the incubation period.

VP-positive yeasts appear to be of no concern in processing frozen concentrated orange juice, since they are few in number as a natural contaminant. However, when an increase in diacetyl occurs in the high-Brix stages of concentration (above 42° Brix), the presence of VP-positive yeasts should be suspected inasmuch as bacteria are of no concern at these Brix levels (Berry *et al.* 1956; Murdock and DuBois, 1955).

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Influence of Buffers and pH on the Thermal Destruction of Spores of Bacillus megaterium and Bacillus polymyxa[a]

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SUMMARY

Spores of *Bacillus megaterium* 1A28 and *B. polymyxa* 1A39 were heated at 100°C in buffers adjusted to different pH values. Generally, recovery of survivors was greatest in the neutral zone. Variations in recovery of survivors were attributable to the organism, buffer constituents, and pH of the buffer system. Use of different phosphate salts had no influence on destruction; stability was greatest in a range of .005—.050*M* phosphate. Citrate, phthalate, or ammonium ion in the buffer usually reduced heat resistance of the spores below that demonstrated in phosphate buffer.

INTRODUCTION

In general, foods having an acid pH can be sterilized in less time and with less heat than foods having a pH in the neutral zone. However, differences observed in the resistance of spores in various heating media cannot be attributed entirely to pH. For example, juices prepared from different foods but of the same pH and inoculated with the same number of spores may require different sterilizing times (Bigelow and Esty, 1920; Esty and Meyer, 1922). Sognefest *et al.* (1948) observed that the lower the pH in the range of 4.5–9, the lower was the heating required for sterilization of puréed food products; but no direct correlation was observed between pH and F_0 values when one low-acid food product was compared with another. They concluded, as did Aschehoug and Jansen (1950), that the heat treatment required to prevent spoilage varied with the type of product as well as with pH. Riemann (1963) observed the influence of NaCl, $NaNO_3$, $NaNO_2$, pH, and F_0 on spore destruction in the canning of meat. All of the factors studied except $NaNO_3$ had a statistically significant effect on the number of spores that could initiate growth. He concluded from his observations that the preserving system in canned cured meats is complex, and that one factor cannot be evaluated without consideration of the others.

Studies on the heat resistance of spores in buffer systems have indicated that, in general, resistance is maximum at neutrality or in a zone bordering neutrality. Murray and Headlee (1931) reported that spores of *Clostridium tetani* suspended in Clark and Lubs' buffer solutions (pH 1–10) showed maximum resistance at pH 7.0. Variation between pH 5.0 and 9.0 had little effect on resistance. In the same buffer system, spores of *Bacillus anthracis* showed greatest resistance at pH 8.0 (Murray, 1931); spores of *Cl. welchii (Cl. perfringens)* showed greatest resistance at pH 5.0 (Headlee, 1931). Esty and Meyer (1922) found that spores of *Cl. botulinum* demonstrated maximum resistance in phosphate mixtures between pH 6.3 and 6.9; and that the optimum for spores of *B. subtilis* was near the neutral point, with no apparent difference between pH 6.8 and 7.6. Similar observations have been reported for spores of *B. cereus* in phosphate buffer (Vas and Proszt, 1955, 1957); for spores of *B. subtilis* (Williams, 1929); and for spores of *Cl. botulinum* (Weiss, 1921). Other examples of the influence of pH on thermal destruction of spores in foods and in buffer systems are

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discussed by Baumgartner and Hersom (1956) and by Schmidt (1957).

The present study was undertaken to determine the effects of different buffering systems and of variation in pH of the buffering system on the destruction of spores of *Bacillus megaterium* and *B. polymyxa*.

EXPERIMENTAL METHODS

Cultures. Cultures of *Bacillus megaterium* 1A28 and *B. polymyxa* 1A39 were obtained from the Department of Bacteriology, Iowa State University. Stock cultures were maintained on a 1:1 mixture of tomato juice agar and stock culture agar (Difco).

Production of spores. Spores of *B. polymyxa* 1A39 were produced in the G medium of Stewart and Halvorson (1953). An inoculum was prepared by washing the growth from the surface of ten stock-culture agar slants and adding this to 1½ L of G medium, which was then incubated overnight at 30°C. These cells were added to 15 L of G medium in a 20-L Pyrex carboy aerated at a rate calculated to be 3.5 L of air per minute. After sporulation, which occurred within 24 hr, the spores were collected by centrifugation.

Spores of *B. megaterium* 1A28 were produced on the surface of tomato juice agar in large Pyrex dishes (8×12×2 inches). Sporulation was considered complete when 90% or more of the cells had sporulated; this required about 4 days. The plates, which had been incubated at 30°C, were then held for 24 hr at 4°C to aid in the lysing of remaining vegetative cells. Growth was washed from the agar surfaces with acidified distilled water (pH 2.0-2.5). The spores were cleaned as described previously by Walker *et al.* (1961).

Buffer mixtures. Buffer constituents were 0.2*M* except citric acid, which was used at the 0.1*M* level. A series of phosphate buffers (KH_2PO_4: K_2HPO_4) was used over a pH range of 5.2–8.3 for initial work with *B. megaterium* 1A28. Appropriate combinations of the following compounds were used to obtain buffer mixtures of pH 5.5, 7.0, and 8.0: KH_2PO_4 and K_2HPO_4; KH_2PO_4 and NaOH; K_2HPO_4 and NaH_2PO_4; and Na_2HPO_4 and citric acid (MacIlvaine's buffer). Mixtures of potassium acid phthalate and NaOH and of acetic acid and sodium acetate were used for additional pH 5.5 buffers; and mixtures of boric acid and NaOH and of NH_4Cl and NH_4OH for pH 8.0 buffers.

Survival of spores in these buffers was determined as described by Walker *et al.* (1961). A minimum of three determinations were made for each survival curve. Measurement of pH before and after heating showed no change.

RESULTS AND DISCUSSION

When spores of *B. megaterium* 1A28 were heated in a series of phosphate buffers ranging in pH from 5.2 to 8.3, destruction was least at neutrality (Fig. 1). Survival was identical at pH 6.5, 7.5, and 8.0, as was survival at pH 5.2, 5.5, and 6.0. The difference between survival at pH 7.0 and at pH 6.5, 7.5, and 8.0 was not great but

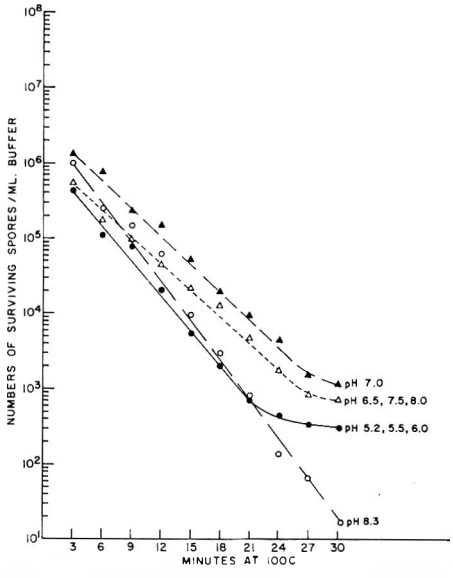

Fig. 1. Survival of spores of *B. megaterium* 1A28 heated in 0.2*M* phosphate buffers (KH_2PO_4: K_2HPO_4) of different pH values.

occurred consistently. As acidity increased beyond this zone, survival was less. Schmidt (1957) has pointed out that resistance of spores is usually at a maximum in the pH range of 6.0–8.0, but the extent of this zone can vary with the particular spores, the suspending medium, and the method of recovery of survivors.

Additional studies were limited to the pH values of 5.5, 7.0, and 8.0. Figs. 2, 3, 4 show survival of spores in various buffers at these levels of acidity. Several combinations of phosphate compounds were used to determine the effect of the different salts on survival. No discernible differences occurred among the various phosphate salts except in the citric acid combination. In the presence of citric acid, survival was always less than in other phosphate-containing systems; nevertheless, number of survivors in this buffer

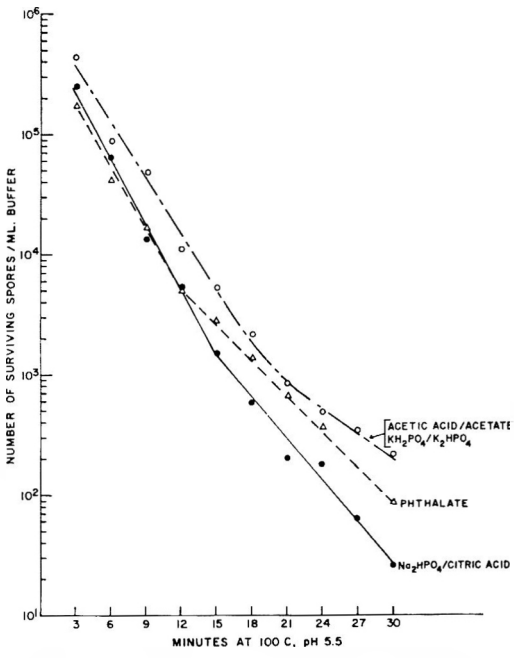

Fig. 2. Survival of spores of *B. megaterium* 1A28 heated in different buffers adjusted to pH 5.5.

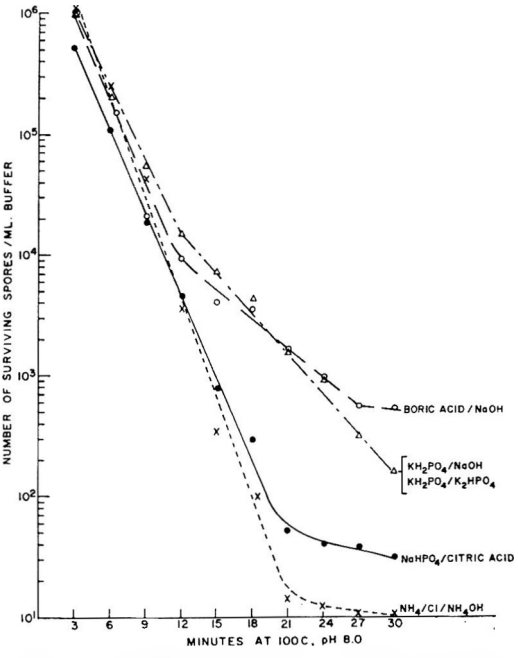

Fig. 4. Survival of spores of *B. megaterium* 1A28 heated in different buffers adjusted to pH 8.0.

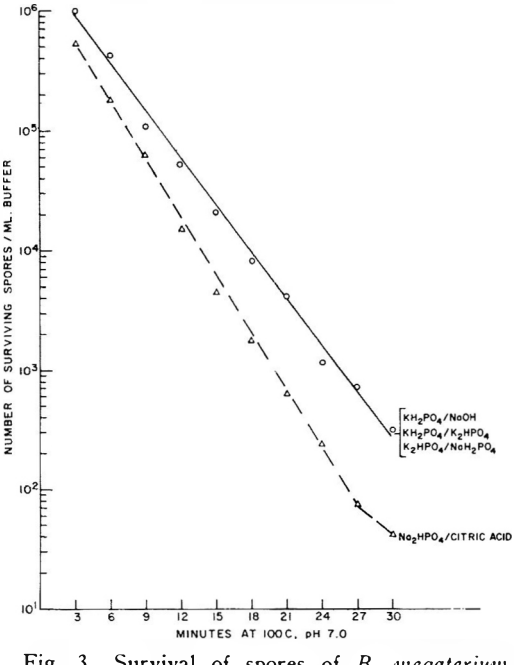

Fig. 3. Survival of spores of *B. megaterium* 1A28 heated in different buffers adjusted to pH 7.0.

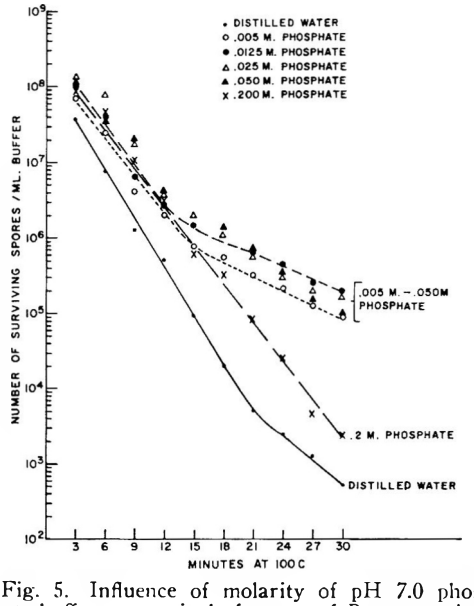

Fig. 5. Influence of molarity of pH 7.0 phosphate buffer on survival of spores of *B. megaterium* 1A28.

system was greatest at pH 7.0. Anderson *et al.* (1949) found that the addition of citric and acetic acids in tomato juice reduced the heat processing necessary to kill *B. coagulans*. In these buffer systems, acetic acid did not have this effect (Fig. 2).

Variation in molarity of the phosphate concentration influenced thermal survival of *B. megaterium* to a limited extent. Kill (Fig. 5) was greatest in distilled water and

in 0.2*M* phosphate buffer. Survival was at a maximum in the range of .005–.050*M* phosphate. Differences in survival at the molarities in this range were considered not to be of consequence. Amaha and Ordal (1957) and Ordal and Lechowich (1958) observed maximum resistance for spores of *B. coagulans* in 0.025*M* phosphate buffer; when the phosphate concentration was increased or decreased, the rate of spore destruction was accelerated. Other workers have also reported that phosphate concentration influences the death rate of spores (Amaha and Sakaguchi, 1954; Williams and Hennessee, 1956). Kittaka and Ordal (1963) have presented evidence that differences in ionic strength may influence the rate of thermal destruction of spores.

Phosphate ions appear to have some protective effect on spores. For example, replacement of phosphate buffer (pH 7.0) with other buffers resulted in a significant increase in rate of kill of *B. coagulans* (Amaha and Ordal, 1957). Licciardello and Nickerson (1963) found that the resistance of *B. subtilis* was greatest in phosphate buffer and least in nutrient broth. Similarly, Levinson and Hyatt (1960) reported that spores of *B. megaterium* survived heating better in phosphate buffer (pH 7.0) than in cacodylate buffer (pH 7.0) or in water. Addition of phosphate protected the cacodylate-suspended spores from the detrimental effects of heat.

As shown in Fig. 4, heat survival was lowest in the NH_4Cl-NH_4OH buffer. Perhaps adsorption of ammonium ions on the spore surfaces caused reduction in heat resistance. Alderton and Snell (1963) observed that spores of *B. megatarium* react as cation-exchange resins and that substitution of ammonium ion on the spore surface decreased heat resistance. Variability in heat destruction in different buffers as well as in different foods might be partially explained on the basis of the cations that are adsorbed on the spore surface. Perhaps the differences in thermal destruction in acetate buffer (pH 5.5), borate buffer (pH 8.0), and phthalate buffer (pH 5.5), other than those related to pH, could be attributed to interaction with cations (Ca^{++}, Mg^{++}, Mn^{++}, etc.) that can influence heat stability.

In further comparisons with both *B. megaterium* and *B. polymyxa*, buffer mixtures were used consisting of KH_2PO_4 and NaOH and of Na_2HPO_4 and citric acid. These two systems have buffering capacity at pH 5.5, 7.0, and 8.0; however, they differ in that survival is less in the presence of citrate. In addition, phthalate buffer, pH 5.5, was used since relative survival in it was similar to that of citrate-containing buffer, pH 5.5. In these comparisons, 10^9 spores/ml of buffer were used rather than 10^7/ml, which had been used previously.

Survival of *B. polymyxa* in these buffer mixtures was greatest at pH 7.0; survival was least in the phosphate-citrate buffer, pH 8.0, and the phthalate buffer, pH 5.5 (Fig. 6). These reactions agree closely with those previously described for *B. megaterium* (Figs. 2, 3, 4) and those presented in Fig. 7. Variation in pH did not seem to affect *B. megaterium* to the extent that it did *B. polymyxa*. No difference (Fig. 7) in survival of *B. megaterium* could be detected in the KH_2PO_4-NaOH buffer, regardless of pH. Previously only small differences between these same acidities were observed (Figs. 2, 3, 4). Perhaps these differences are not pronounced when higher numbers of spores are used; in this instance, 10^9 spores/ml vs. 10^7 spores/ml.

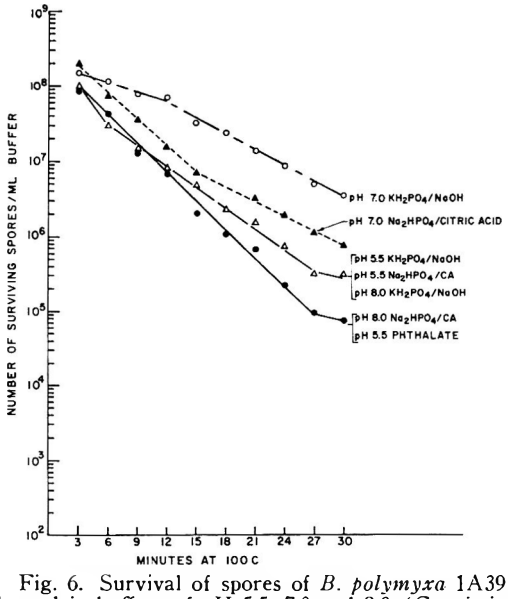

Fig. 6. Survival of spores of *B. polymyxa* 1A39 heated in buffers of pH 5.5, 7.0 and 8.0 (Ca, citric acid).

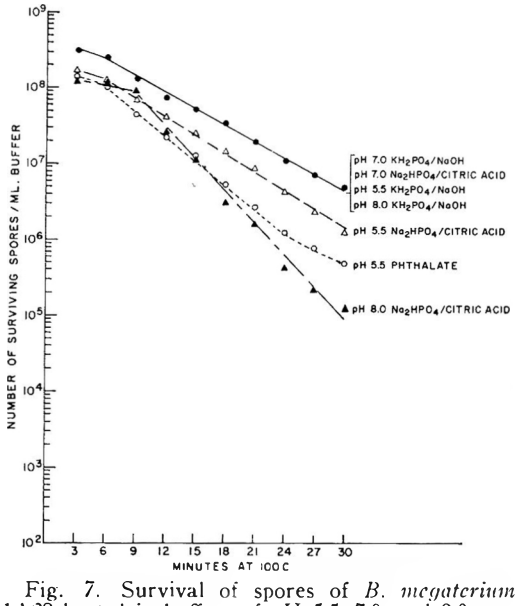

Fig. 7. Survival of spores of *B. megaterium* 1A28 heated in buffers of pH 5.5, 7.0, and 8.0.

Undoubtedly, pH influences the destruction of spores, but other factors also can alter the outcome. A solution of pH 7.0 in one buffer system may not be any more favorable for survival than an acid or alkaline solution in another buffer system; however, if comparisons are made within a given buffer system, pH 7.0 or at least a zone around neutrality usually affords best survival.

The changes taking place during killing of the spore are largely unknown. Ball and Olson (1957) have related death of spores to the velocity with which molecules or ions in the environment are moving. They proposed that the hydrogen ion is more effective than the hydroxyl ion, because of its lighter weight and greater speed. Also, pH could affect the ions present in the spore's environment and influence the type that would be adsorbed on the surface, which in turn would alter heat stability (Alderton and Snell, 1963). Amaha and Ordal (1957) offered the explanation that differences in rate and degree of thermal destruction might be related to the loss of one or more of the cations which contribute to the thermal resistance of spores and that the rate of loss of viability would be greater in the presence of increased phosphate concentration or in the presence of more powerful or more specific chelating agents. None of these proposals, however, explains the actual mechanism of modified heat stability of the spore and subsequent death.

The influence of specific environmental factors on destruction of spores can be more easily observed in buffer systems than in foods. In foods, for example, salt, depending on the concentration, can increase or decrease the amount of heat required for destruction of spores (Anderson *et al.*, 1949; Esty and Meyer, 1922; Headlee, 1931; Murray and Headlee, 1931; Viljoen, 1926). The presence of organic matter can also afford protection to spores during heating; for example, peptone, albumin, nucleic acids, sugars, and starch can provide protection at certain concentrations (Amaha and Sakaguchi, 1954; Anderson *et al.*, 1949; Headlee, 1931; Murray, 1931; Murray and Headlee, 1931; Weiss, 1921). Knowledge of the effect of various buffer systems and acidity on spore destruction will ultimately help in evaluating the role and importance of such components of foods as salt, sugar, proteins, fats, and other inorganic and organic materials in the sterilizing process.

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Irradiation-Induced Textural Change in Fruits and Its Relation to Pectin Metabolism[a]

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SUMMARY

Textural changes of pears and peaches exposed to gamma radiation of 300, 600, and 900 Krads were found to correspond to a decrease in protopectin content and an increase in pectin and pectate fractions of the fruits. Characterizations of the 70% alcohol-insoluble solids prepared from these fruits revealed only minor differences in respect to their anhydrouronide and acetyl content and degree of esterification. To account for the marked effects of radiation, activities of pectic enzymes extracted from irradiated fruits were investigated. Pectin methylesterase showed an increased activity immediately after doses of 300 and 600 Krads, and enzymes extracted from fruit four days after irradiation had a reduced activity. Pears, peaches, and nectarines irradiated under nitrogen atmosphere softened much less than fruits irradiated under air. While this may be attributed to the reduced formation of free radicals under anoxic conditions, a further experiment with pectin solutions exposed to gamma radiation under similar atmospheric conditions did not support this suggestion. The changes in 1% pectin solutions were related to radiation dose, but were remarkably similar regardless of the presence or absence of oxygen during exposure.

INTRODUCTION

Softening of fruits and vegetables after ionizing radiation has been observed and reported (Glegg *et al.*, 1956; Boyle *et al.*, 1957; Clarke, 1959; Maxie, 1963). It has also been postulated that the softening that occurs during normal ripening is related to the decomposition of insoluble pectin compounds of the middle lamella (Kertesz, 1951; Ulrich, 1958). Since chemical changes in macromolecular polymers, such as the pectin, have been shown to occur after exposure to ionizing radiation (Kertesz *et al.*, 1956), the softening effect of irradiation might well be the result of the degradation of pectins *in situ*.

Viscosity measurements of solutions made from irradiated pectin powder indicated a degradation of pectin materials (Kertesz *et al.*, 1956). To date, however, the work of McArdle and Nehemias (1956) has been the only report on the decomposition of pectins extracted from irradiated fruit. They found a relation between radiation-induced softening of apple and carrot tissues and the changes that took place in the various pectin fractions. Unfortunately, the radiation facilities available then did not possess controls for such important conditions as temperature and atmosphere during exposure. The time interval between radiation treatment and chemical or physical analyses was not always adequately specified. Our preliminary investigations indicated, however, that treatment parameters may have a marked influence on textural changes. Specification of the length and conditions of storage after radiation treatment is also important, since further textural changes may take place during this period (Maxie, 1963).

The installation of a Mark II-type cobalt-60 gamma source has made it possible to control most of the treatment variables adequately (Romani *et al.*, 1963). The study reported here investigated textural changes of fruits and their relation to changes in pectin substances and pectic enzymes. Various fruit species and model systems were used as experimental materials, which were maintained under controlled conditions during and after irradiation.

MATERIALS AND METHODS

The fruits were obtained from local sources. They were harvested at commercial maturity and stored immediately at 32°F until radiation treatments. Following irradiation they were prepared

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for pectin analysis either immediately or after four days at 68°F.

Pectin solutions were prepared from citrus pectin (Sunkist Growers, Inc. D. 3547) and irradiated in sealed vials.

Irradiation was performed in a Mark II-type, 32,000-curie, cobalt-60 food irradiator (Romani *et al.*, 1963) that provided a gamma exposure of approximately 300 Krads per hour. Dosimetry had been determined by the Fricke dosimeter, which indicated that dose distribution was within ±8%. Temperature was maintained at ca. 65°F during irradiation. Fruits were irradiated in a closed container in which air or nitrogen was circulated prior to and during irradiation at a rate of approximately 200 ml/min, with the application of the system designed by Claypool and Keefer (1942) for respiratory studies.

Firmness changes were measured on both sides of each fruit with the Magness-Taylor pressure tester equipped with a 5/16-inch plunger. Pressure testers with a range of either 0–30 lb or 0–10 lb were used depending on the firmness of the fruit.

The method used for extraction and preparation of the various pectic fractions was largely adapted from the procedure outlined by Kertesz (1951). This analysis is based on the solubility properties of pectin and premised on the fact that the ease of solution of pectin in water increases with decreasing length of the pectin molecule.

A 500-g sample of peeled tissue was taken from at least 10 fruits in each treatment. The fruit tissues were extracted with boiled ethyl alcohol maintained at a concentration of not less than 70%. The dried and pulverized sugar-free material, referred to herein as "alcohol-insoluble solids" (AIS), was extracted by mild means as follows: a) twice with water at room temperature to remove pectin; b) twice with 0.5% ammonium oxalate at room temperature to remove pectate; c) twice with 0.05*M* HCl at 95°C to remove protopectin. Pectins were precipitated from the extracts by adding two volumes of 95% ethanol and sufficient HCl to make them 0.05*M* of the total volume for each. The combined figures for a, b, and c were used as "total pectin."

The precipitates were collected with centrifugation at approximately 12,000 × G, using a continuous-flow system attached to a Servall centrifuge, washed several times with acetone, dried, and weighed.

Degree of esterification, acetyl content, and anhydrouronic acid content of the pectin molecules were determined from AIS preparations following the method described by Gee *et al.* (1958).

Polygalacturonase was prepared from 200 g of peeled fruits, following the method described by Hobson (1962). Enzyme activity was determined at pH 5.0 on a pectic acid substrate by measuring the increase of reducing power; a 1-ml substrate-enzyme mixture was taken out at 10-min intervals from each sample, and reducing groups were determined with the picric acid method (Hobson, 1962).

Pectin methylesterase was prepared from 50 g of mature chilled and pitted cherry fruits to which were added 50 ml 1.0*M* sodium acetate followed by comminution for 1 min in a Waring blender at full speed. After adjusting the pH to 8.0 the extraction was continued for 2 hr with continuous stirring. The samples were then centrifuged 15 min at 5,000 × G and the supernatant used as the enzyme source. All extraction procedures were carried out at 0–2°C. The nitrogen content of the enzyme sources was determined by standard Nessler techniques. Methods described by Rouse and Atkins (1955) were used for the enzyme assay. In brief, 5 ml of enzyme extract was added to 50 ml of 1% pectin solution containing 0.2*M* NaCl and held at 30°C. The pH of the reaction mixture was adjusted to 7.5 and maintained there for the 30-min reaction period by adding 0.05*N* NaOH. Results are expressed in pectin methylesterase units, which represent the meq of ester hydrolized per minute per mg of enzyme nitrogen.

Changes in the irradiated 1% pectin solutions were analyzed by measuring reducing groups and viscosity. Reducing groups were determined with the picric acid method (Hobson, 1962), and viscosity changes were detected in a No. 300 Ostwald-Cannor-Fenske viscometer at 30°C. Results are expressed as percent change relative to the air control.

RESULTS AND DISCUSSION

Effect of irradiation in air on texture. Table 1 shows pressure-test data for pears and peaches irradiated under air at 300, 600, and 900 Krads. These results clearly show the immediate softening effect of ionizing radiation on fruits. After four days the control peaches became so soft in the course of normal ripening that no differences among treatments could be detected with the method

Table 1. Firmness of fruits immediately and four days after radiation (average results for 20 fruits).

	Firmness (lb pressure)			
	Peach (Rio Oso Gem)		Pear (Bartlett)	
Dose (Kilorads)	0 days	4 days	0 days	4 days
0	4.8	1.2	14.2	3.1
300	1.5	1.1	7.5	6.0
600	1.5	1.2	4.8	5.4
900	1.3	1.1	4.8	2.8

used. With pears, however, recovery in firmness was noted in some irradiated fruit as well as a delay in the rate of softening associated with normal ripening. The highest dose resulted in a brown breakdown, and fruits lost their consistency completely.

Similar results were reported earlier by Hannan (1955), Clarke (1959), and Maxie (1963). These data (Table 1) are presented to characterize the tissues on which the subsequent analyses were performed.

Effect of irradiation in air on chemical changes in pectin fractions. Chemical analyses of pears showed a slight fall in total pectin content as the dose proceeded to the maximum (Fig. 1). HCl-extractable pectin (i.e. protopectin) content, however, decreased considerably with increasing doses immediately following radiation, whereas water-soluble fraction (i.e. pectin) increased to about twofold, and ammonium-oxalate soluble fraction (i.e. pectate) increased only slightly. Four days after radiation treatment the protopectin content of the 300- and 600-Krad treatments was about 60% higher than that of the untreated fruits. Since, after this storage period, untreated pears became softer than 300- and 600-Krad-treated fruits (Table 1), these textural changes corresponded to solubilization of the protopectin fraction. At the highest dose, which resulted in cell necrosis and breakdown, the fruits were much softer than the controls and contained less protopectin and more of the soluble pectin fractions.

As with pears, irradiated peach fruits also exhibited a very slight decrease in total pectin content (Fig. 2). The HCl extract, however, showed an immediate sharp decrease following irradiation, and the magnitude of the change increased with radiation dose. At the same time, there was a slight increase in the water-extractable solids, which represent the pectin fraction with the shortest chain length. Similar analyses carried out four days later showed only minor changes in total pectin. The HCl-extractable fraction of the untreated fruits decreased almost to the level that was found with irradiated fruits.

These changes in pectin fractions seem to follow the pattern reported for the softening

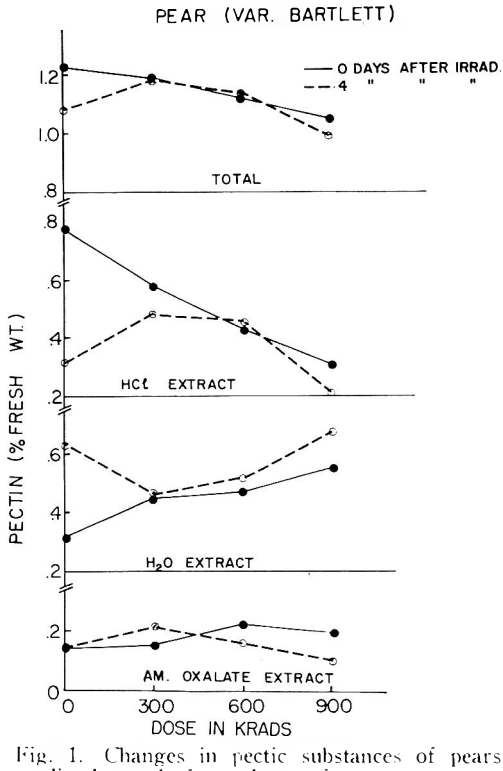

Fig. 1. Changes in pectic substances of pears immediately and four days after exposure to gamma radiation.

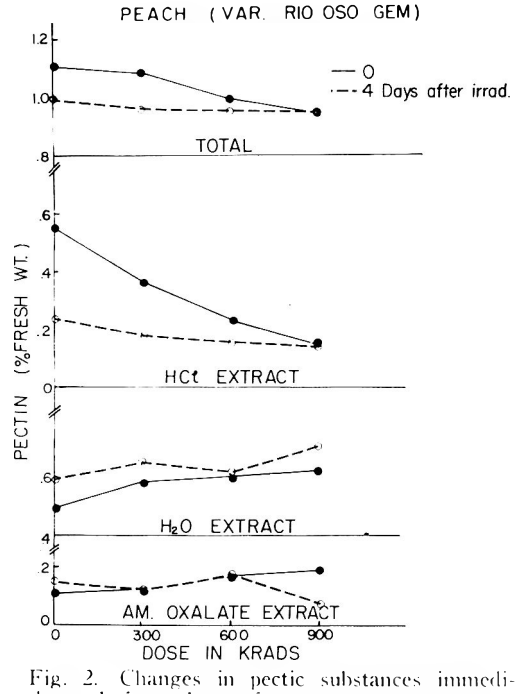

Fig. 2. Changes in pectic substances immediately and four days after exposure to gamma radiation.

of fruits during normal development (Postlmayr *et al.*, 1956; Woodmansee *et al.*, 1959; Esau *et al.*, 1962). It was found that fruit development is accompanied by an increase in water-soluble pectin substances and a decrease in protopectin content. To date, however, complete quantitative data on the transformation of protopectin into pectins are limited even for the normal changes in fruits.

Seventy percent alcohol-insoluble solids, degree of esterification, acetyl content, and anhydrouronide content were determined from irradiated pear and apple fruits immediately and four days after radiation treatments. These results are shown in Table 2. There was a slight decrease in AIS and in degree of esterification, and an increase in acetyl content, though by no means comparable to the extent of changes reported by others. McCready and McComb (1954) found that the degree of esterification decreased from 89 to 43% during the ripening of pears. With peaches, however, they found only a small difference between unripe and ripe fruits in this respect (89 vs. 86%). Woodmansee *et al.* (1959), working with apples and tomatoes, found that, during the normal process of ripening, while protopectin is being broken down and appears in the water-soluble form, the AIS and degree of esterification markedly decrease and acetyl content rises. That these pronounced changes occur during the ripening of fruit is generally accepted (Ulrich, 1958). Doesburg (1957), however, found little change in the degree of esterification of pectins during the ripening of apples although there was an increase in soluble pectin content. Our results with radiation-induced softening of fruit tissues seem to agree with this later report.

Effect of irradiation in air on pectic enzymes. Degradation of pectin by pectic enzymes has also been reported to have marked effects on the texture of fruits (Bell *et al.*, 1950; Steele and Yang, 1960). To investigate the mechanism of the radiation-induced degradation of protopectin, an attempt was made to determine the activity of pectic enzymes extracted from irradiated fruits.

Polygalacturonases have not been widely reported in fruits other than tomatoes and avocados (McCready *et al.*, 1955). Samples of pears, peaches, and cherries were extracted for this enzyme. No polygalacturonase activity, as measured by an increase in reducing power in the substrate, was found in the fruit samples analyzed. Weurman (1953) reported that an inhibitor occurs in some varieties of pear, and is undoubtedly present in some other fruits as well, which may prevent the action of polygalacturonase.

On the other hand, pectin methylesterase (PME) has been easily prepared from many fruits (Lineweaver and Jansen, 1951). This enzyme could be a controlling factor in pectin solubilization. It has been postulated that de-esterification by PME must precede any glycosidic hydrolysis of pectin by polygalacturonase (Jansen and McDonnel, 1945).

Lower radiation doses resulted in an increase in PME activity when extracted immediately after the exposure (Table 3). Enzyme assays four days after radiation showed a considerable reduction in PME activity. An increased rate of enzymatic

Table 2. Yield and pectin analyses of alcohol-insoluble solids prepared from irradiated fruits 0 and 4 days after irradiation.

	AIS (% fruit)		Anhydrouronic Acid (% of AIS)		Acetyl (% of AIS)		Degree of esterification (% of AIS)	
Sample	0 days	4 days	0 days	4 days	0 days	4 days	0 days	4 days
Pear (Bartlett)								
Control	2.72	2.72	27.5	27.6	1.4	1.9	74.3	72.6
300 Krad	2.72	2.70	26.1	25.9	1.5	2.0	73.0	69.4
600 Krad	2.54	2.52	24.1	24.3	2.0	2.4	71.8	65.2
Apple (Rome Beauty)								
Control	1.88	1.78	23.6	23.6	0.6	1.4	71.3	70.5
500 Krad	1.80	1.64	23.4	22.7	0.7	1.4	71.4	69.0

Table 3. Pectin methylesterase activity of irradiated cherries 0 and 4 days after exposure.

Dose (Kilorads)	PME activity PME (units/mg N) × 10^4 0 days	4 days
0	19.0	17.3
200	26.0	19.7
500	23.0	17.7
1000	16.0	12.0

activity may thus be partly responsible for the initial pectin degradation.

Effect of irradiation in nitrogen atmospheres. Pears, peaches, and nectarines exposed to ionizing radiation softened much less under nitrogen atmosphere than when exposed to similar doses in air. Our preliminary studies indicated that to achieve this effect the fruits required more than 24 hr of nitrogen treatment prior to irradiation. Accordingly, fruits used in this experiment were placed under air or N_2 atmospheres 36 hr prior to and during the radiation exposure. The pre-radiation temperature was 68°F for the peaches and 50°F for pears and nectarines. Table 4 shows pressure test data for the fruits immediately after radiation. Softening of peaches was reduced under N_2 even for the controls, possibly from retarded or abnormal ripening. The oxygen-free atmosphere prevented radiation-induced softening to a certain degree with all doses used. This may be explained by a reduced formation of free radicals under this condition. However,

Table 4. Firmness of fruits after irradiation under air or nitrogen atmosphere.

Sample	Firmness (lb pressure)[a] Air	N_2
Pear (Bartlett)		
Control	22.4	22.4
300 Krad	15.1	19.4
900 Krad	8.2	11.1
Peach (Red Globe)		
Control	6.4	15.4
300 Krad	3.0	7.4
900 Krad	2.2	5.1
Nectarine (Late Le Grande)		
Control	14.0	14.9
300 Krad	5.7	8.4
400 Krad	4.8	6.6

[a] Average readings from samples of 15 pears, 15 nectarines, and 20 peaches.

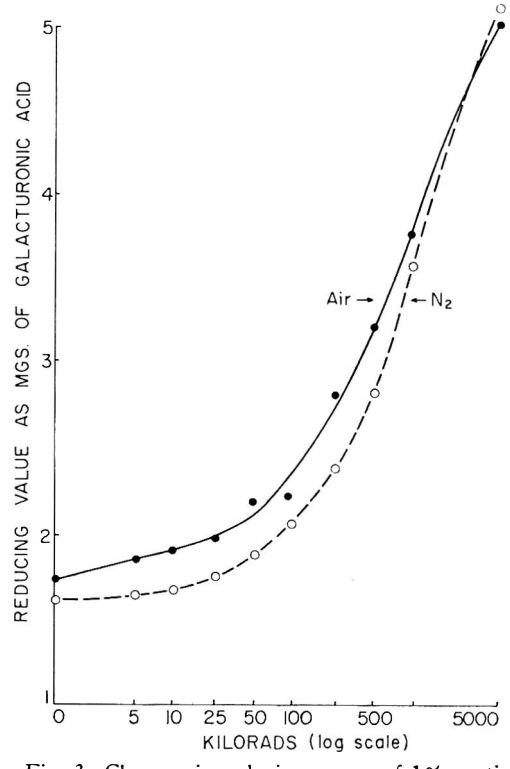

Fig. 3. Changes in reducing power of 1% pectin solutions after exposure to gamma radiation under air or nitrogen atmosphere.

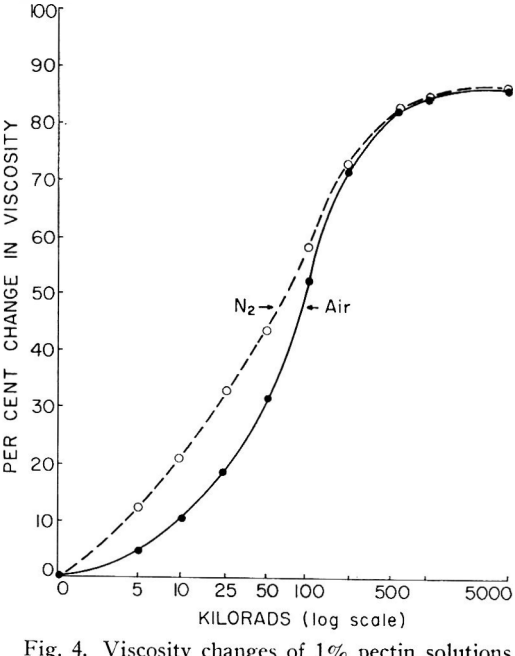

Fig. 4. Viscosity changes of 1% pectin solutions after exposure to gamma radiation under air or nitrogen atmosphere (percent change relative to the air control).

results with 1% pectin solutions irradiated under air or nitrogen (Figs. 3 and 4) showed that although changes were directly related to radiation dose they were remarkably insensitive to the presence or absence of oxygen during exposure. This suggests that radio-chemical changes in the pectin, as indicated by reducing power or viscosity, are direct radiation effects rather than mediated by oxidizing radicals.

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An Electronic Analog for the Olfactory Processes[a]

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SUMMARY

Polarized microelectrodes were utilized as sensing devices, analogous to the human olfactory hairs, for qualitative and quantitative responses to volatiles. The measured response current is attributed to the interaction of a volatile with an electrical double layer at the electrode interface. The use of different microelectrode metals, electrolytes, and impressed voltages makes possible different electrical double-layer phenomena. With the use of only a few of the many possible combinations of electrode metals, electrolytes, and impressed voltages, differential-response sensitivity was obtained for a variety of volatiles at any given microelectrode condition. A comparison of the responses of several odorants at a number of different electrode conditions demonstrated a differential-response specificity similar to that thought to occur in the human at different olfactory receptor sites.

INTRODUCTION

Our basic knowledge of the human olfactory mechanism has been impeded inherently by anatomical inaccessibility to the olfactory region. We therefore have more theories of diversified merit than facts on the olfactory mechanism. Work accomplished with experimental animals, though valuable, is still generally accepted as limited.

Olfaction is a process requiring adsorption. The interaction of the odorant molecule with the olfactory receptor surface is the stimulus responsible for triggering the nerve impulse, but the nature of this interaction has not yet been elucidated.

After consideration of the various theories and available facts, Hartman (1954) introduced a concept of physical nature that might be involved in olfactory perception and exploited this idea with an electronic analog that resulted in responses to odorants. Further work on this instrumentation was later reported by Hartman and Tolle (1957). The present paper reports the results of recent studies and modifications of the prototype of an electronic analog for the olfactory processes.

Theoretical considerations. Adsorption has no strictly confined limits of definition. There is no sharp demarcation between a weak and a strong van der Waals force and a weak hydrogen bond, or between a strong hydrogen bond and a weak valence link. Adsorption, in any case, implies that there must be some change in the internal energy of the molecules involved. Adsorption may, therefore, also be likened to a very weak oxidation-reduction process in that the molecular electron orbitals undergo shifts from their mean dynamic equilibria, although gain or loss of electrons is not necessarily involved for this energy change.

The internal energy of the molecule is in part related with the shared inter-atomic electrons, and therefore with the bonding angles between the atoms, which are permitted certain degrees of freedom. Odorant molecules, on contact with the olfactory surface, undergo rearrangement of the electron orbital equilibria, with consequent modifications of the bonding angles, and therefore adjustments in potential or vibrational energy. Since the bonding angles are permitted certain degrees of freedom, energy modifications are likewise variable, although somewhat specific for the individual molecule under any given condition. We may therefore assume that either part or all of the energy for stimulation of the olfactory receptors is probably derived from the odorous molecule.

If the adsorption of odorant molecules on the olfactory receptors results simply in heat evolution (heat of adsorption), then

[a] This investigation was supported in part by Public Health Service Research Grant EF 00262 from the National Institutes of Health.

olfaction may be analogous to a thermistor receptor system. It is very improbable that the olfactory receptors perform in this manner, even though it is possible to construct thermistors with differential specificity to odorous molecules. Moncrieff (1961) demonstrated the feasibility of coating thermistors with various substances to produce differential odorant adsorption, which was measured as heat of adsorption.

If adsorption is synonymous with an enzyme-odorant substrate reaction, then we must account for extremely high enzyme turnover numbers of many enzymes with more or less restricted specificity. Although such enzyme systems are possible for certain odorous molecules, the theory would not seem to hold for the thousands of odorous molecules that exist.

To elucidate the transducer system that will account for all or at least most of the characteristics associated with odor sensation, we must consider all the following factors, based on an initial adsorption phenomena:

a) change in potential of the olfactory receptor upon odorant stimulation

b) differential specificity of the receptors for various odors

c) extremely rapid conversion of the stimulus to the nerve impulse

d) a quantitative relationship between stimulus and response that conforms with olfactory perception.

Hartman (1954) was the first to postulate that the olfactory receptor hairs essentially act as polarized microelectrodes. The olfactory hairs, surrounded or projecting through a mucous layer, are under a bioelectric potential, but little current flows along or across the nerve membrane in the absence of odorous molecules. The interface of the mucous and the olfactory hair is the site of olfactory sensitivity and also the site of a polarization phenomenon. More specifically, the polarization phenomenon may be attributed either in part or almost totally to the presence of an electrical double layer (Helmholtz double layer), with properties dependent upon biochemical differences at the receptor site and the bioelectric potential across the receptor membrane.

Hartman (1954) developed an analog to produce this postulated mechanism in the form of a microelectrode. Differential odorant specificity was achieved by variation of the nature, size, and shape of the microelectrode, the applied e.m.f., and the nature and concentration of the electrolyte associated with the system. The polarization that results from this system may be attributed primarily to an electrical double layer and accumulated electrolytic products or gas films at the electrode-electrolyte interface. The area of sensitivity to odorants is greatest at the electrode-electrolyte-air interface, where odorants can make almost direct contact with the double layer without the requirement of dissolving in the aqueous electrolyte and migrating considerable distances by diffusion to the electrode surface.

The electrical double layer may be treated mathematically as a capacitance or energy storage mechanism. Adsorption of odorous molecules at this double layer probably results in either disruption or enforcement of the double layer, resulting in a change in potential and current flow across the double layer. Where nascent or molecular films of O_2 or H_2 exist at the electrode interface, similar disruptive effects may also take place. The microelectrode does not perform as a true capacitor, but more like a leaky condenser.

In the olfactory receptor the electrical double layer may be composed of an activated biochemical structure, serving as the solid phase, and an electrolytic mucus, as the diffuse phase. Aside from the fact that the receptors are composed of "lipoids," little is known of the chemical structures or compounds present. It may be possible that the biochemical receptor surface reacts in a manner analogous to a ferro-electric lattice, as suggested by Dravnieks (1962). The work by Amoore (1962) on stereochemical configuration of receptor sites may also fit in with polarization phenomena, where the receptor sites serve as the fixed or solid portion of an electrical double layer and at the same time render some order of specificity to odorants. Either of these theories would not rule out polarization phenomena as part of the olfactory transducer system. In all probability there

is no one theory existing today, including that which has been proposed here, that offers a true picture of the phenomena involved in olfaction.

INSTRUMENTATION

The original olfactory analog prototypes were discussed in previous publications (Hartman, 1954; Hartman and Tolle, 1957). The present system (Fig. 1) differs in odorant presentation, electrode cell geometry, and electrode design.

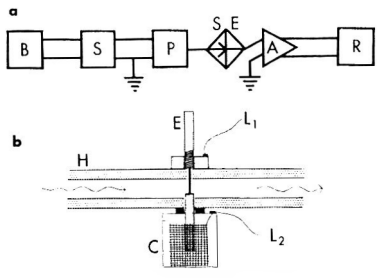

Fig. 1. Olfactory analog instrumentation. a) Electronics system. *B*, 1.5 volt battery; *S*, polarity switch; *P*, potentiometer; *SE*, sensitive electrode system; *A*, amplifier; *R*, recorder. b) Sensitive electrode system. *H*, electrode housing of 8 mm ID tubing carrying moisture-saturated air and odorants to electrode; *E*, microelectrode affixed to threaded brass rod; *C*, electrolyte container with platinum mesh wire (or foil) macroelectrode and porous filter in contact with microelectrode; L_1, L_2, electrode leads. Entire system is shielded to ground.

The voltages impressed on the microelectrode ranged from 0 to about ±1.5 volts. The particular limit of voltage depended on the nature of the metal electrode, area of electrode-electrolyte contact, composition of electrolyte, and electrolytic concentration. These factors influence the potential at which electrolysis of water resulted in background noise inhibitive to accurate measurements.

The area of microelectrode contact with electrolyte varied from 10^{-5} to 10^{-3} square inches. The area of macroelectrode-electrolyte contact was kept at least 100,000 times as great, or generally larger than one square inch, to ensure polarization only at the microelectrode.

The microelectrodes consisted of metallic wires or tubes. The microelectrode tips were shaped by grinding and polishing to a desired form. Although an attempt was made to use variously shaped electrode tips, it was difficult to reproduce many shapes when metal wires of about .005 inch diameter were used. Consequently, the tips used were right cylinders in form.

The electrolytes and electrolytic concentrations employed provided for great flexibility in the state of polarization attained. The only restrictions were that the metal cation should not be discharged under the various conditions, nor should the electrolyte dissolve the respective electrodes. Since some metals have a tendency to dissolve under certain anode conditions, the possibilities of using positive voltages on microelectrodes were restricted. The macroelectrode usually consisted of platinum, which was quite stable under anodic conditions with the electrolytes employed. Electrolyte concentrations ranged up to 1.0 molar, but 0.1 molar was satisfactory for most electrolytes of high conductivity.

A porous glass filter of rod or disc form provided a mechanical stop for reproducible electrode positioning. The porosity and uniformity of the filter affected the height of the meniscus on the electrode, and consequently the final equilibrium polarization. A pore size of 1–2 μ was most satisfactory, and was used in the experiments reported. The electrolyte in the glass filter was above the height of the electrolyte reservoir and was maintained in position by capillary forces in the glass filter.

Deodorized air served as a carrier gas for presentation of the volatiles to the electrodes. This air was moisture-saturated to prevent any rapid changes in electrolyte concentration at the surface of the filter. Such concentration might result in salt creeping or affect the stability of the electrode condition.

Odorant samples were introduced by syringe injection, upstream of the microelectrode, or through a gas-sampling valve as employed in a gas-liquid partition chromatography apparatus.

The resistance of some of the electrolytic cells was in the range of 20,000 ohms. When a steady-state polarization was attained, the resistance was often in excess of 1 megohm.

METHODS

Various combinations of metal electrodes, electrolytes, and impressed voltages were tested in a program to determine the influence of these factors on sensitivity to various odorants with the single electrode system (Fig. 1). The specific electrode conditions are presented with each group of results.

Odorant sampling. In cases where commercially available compounds of high purity were tested, a 1-cc sample of the saturated headspace above the odorant at 70–75°F was presented to the electrode by syringe injection into the air carrier system. The air-carrier flow rate was an important determinant of response amplitude. An air velocity of 440 cm per minute was optimum for the cell geometry. Where raw or processed foods were sampled, two procedures were used. The materials were usually ground 1–3 min in a Waring blender, and a 1-cc sample was obtained with a

syringe through a polyethylene film cover. Another procedure utilized a special stainless-steel blending jar coupled with an air pump and gas-sampling valve. This formed a closed system where the air and volatiles were constantly recirculated through the Waring blender for the duration of the sampling period. A 2-min blending, with the air pump operating, usually sufficed to provide equilibrium odorant headspaces. For raw products a small amount of water was added to speed blending. Canned foods in a liquid pack did not require additional water.

To test the variation of odorant concentration on electrode response, 1 cc of saturated headspace was diluted in a clean odorous Pyrex flask of known volume. This headspace was then diluted by the same procedure except that the flask was warmed to prevent dilution errors from odorant adsorption on the glass surface.

RESULTS AND DISCUSSION

The effect of voltage on response to an odorant with a platinum microelectrode (Figs. 2, 3) resulted in a curve characteristic for the odorant under the given electrode conditions. Such curves are designated voltage oxidation-reduction profiles (VORP). Responses lower than 1×10^{-10} amps are not reported, because of equipment limitations. These profiles take into consideration response amplitude only, and do not reflect differences in shape of response curves (Fig. 4) which resulted from the different applied potentials. The effect of electrolyte on VORP is also evident (Fig. 2).

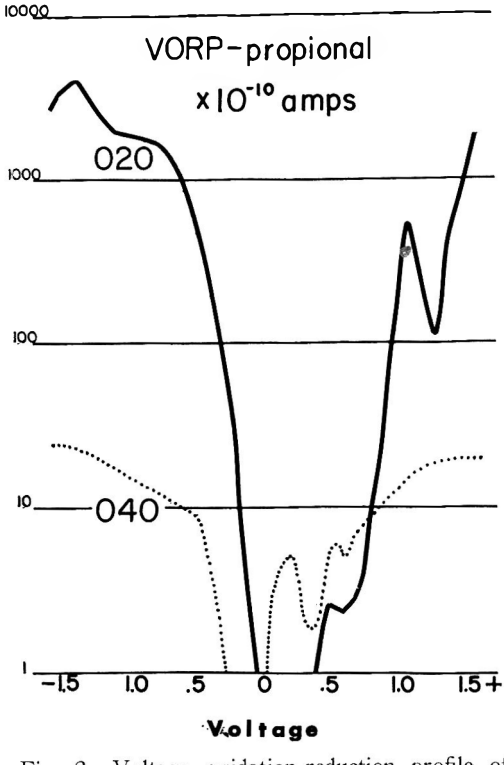

Fig. 2. Voltage oxidation-reduction profile of propional. Electrolyte #020, 0.1*M* $NaHCO_3$ + 0.1*M* Na_2CO_3; electrolyte #040, deionized water with 0.2 ppm NaCl equivalent conductivity; microelectrode, 0.005-inch diameter Pt wire; macroelectrode, Pt; odorant sample, 1 cc saturated headspace at 75°F; airflow velocity, 440 cm/min.

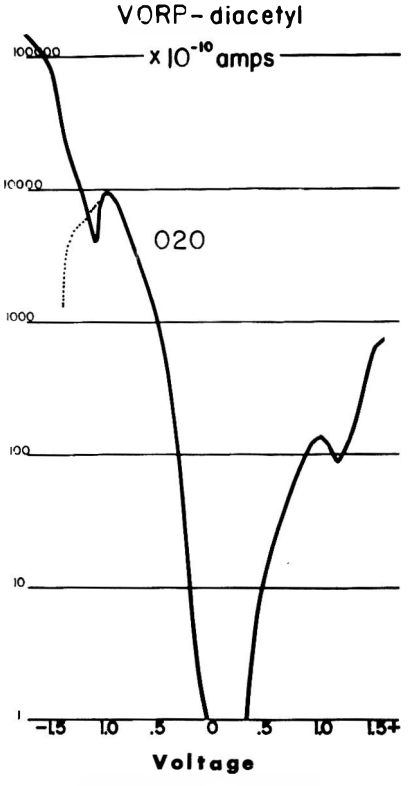

Fig. 3. Voltage oxidation-reduction profile of 2,3-butanedione (diacetyl). Electrolyte #020 and other conditions are the same as Fig. 2. Dotted line represents negative response amplitude following positive deflection.

The effect of the electrode metal on response amplitude (Fig. 5) illustrates the differential specificity that may be obtained with the electronic olfactometer system. Measurements or comparisons of response amplitudes among different odorants do not take into account the effect of metal on shape of response curve (Fig. 6), which is a factor difficult to quantitate but also dangerous to ignore. A comparison of Ni and

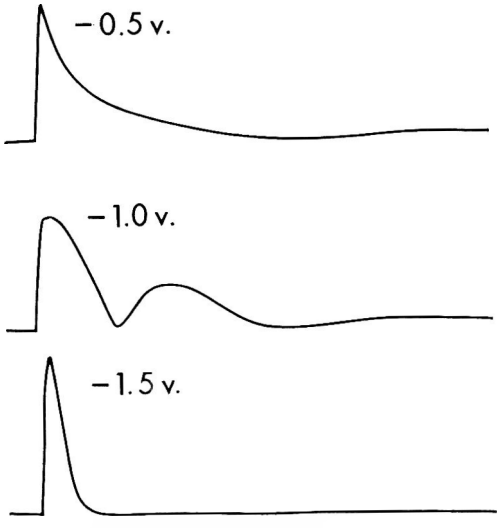

Fig. 4. Effect of potential on shape of response curve. Response attenuation ratio differs at each potential shown. Electrolyte, 0.1*M* $NaHCO_3$ + 0.1*M* Na_2CO_3; microelectrode, 0.005-inch-diameter Pb wire; macroelectrode, Pt; odorant sample, 1 cc saturated diacetyl headspace at 75°F; air flow velocity, 440 cm/min.

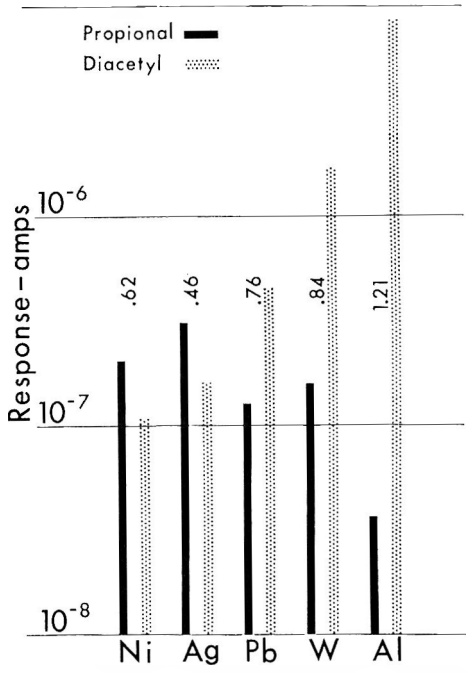

Fig. 5. Effect of electrode metal on response amplitude at polarization current of 6×10^{-7} amps. The applied negative voltage to obtain this current is shown above for each metal. One cc of saturated odorant headspace at 75°F was sampled with an airflow velocity of 440 cm/min.

Al (Fig. 5) shows that propional gave twice the response of diacetyl at the Ni electrode, and diacetyl produced 230 times as great a response as propional at the Al electrode.

To illustrate the differential specificity that may be obtained, a platinum microelectrode was used with 5 electrolytes and 7 voltages to produce 35 electrode conditions. These resulted in a response profile for ethyl sulfide as shown in Fig. 7. These same 35 conditions were used to obtain profiles for 9 different odorants (Fig. 8). The use of a different microelectrode metal would have resulted in profiles different from those illustrated (Fig. 8) even if the combination of electrolytes and voltages was the same. Such a profile method might be employed for identification of pure compounds. Values above 5 (5^{+}, 5^{++}, 5^{+++}) were not plottable on the linear scale employed. Some values indicated by 5^{+++} were 10 to 100 times as great as the quantitative maximum limit of the scale. These profiles, again, take into consideration only response amplitude, whereas additional information may be gained from response curve shapes for different odorants under identical electrode conditions (Fig. 9).

The response to propional at a typical electrode condition as a function of concentration (Fig. 10) assumed a nearly linear relation on a log-log plot. The deviation from linearity at high concentrations may be due to molecular interference at the reactive

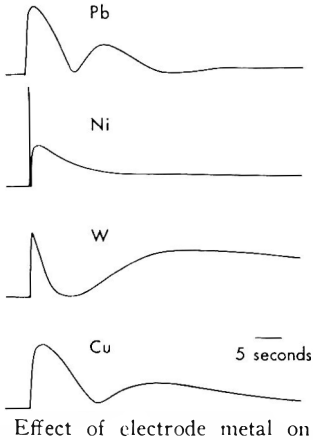

Fig. 6. Effect of electrode metal on shape of response curve. Response attenuation ratio differs for each metal. Electrolyte, 0.1*M* $NaHCO_3$ + 0.1*M* Na_2CO_3; potential, −1.0 volt; microelectrode diameter, 0.005 inch; macroelectrode, Pt; odorant sample, 1 cc saturated diacetyl headspace at 75°F; airflow velocity, 440 cm/min.

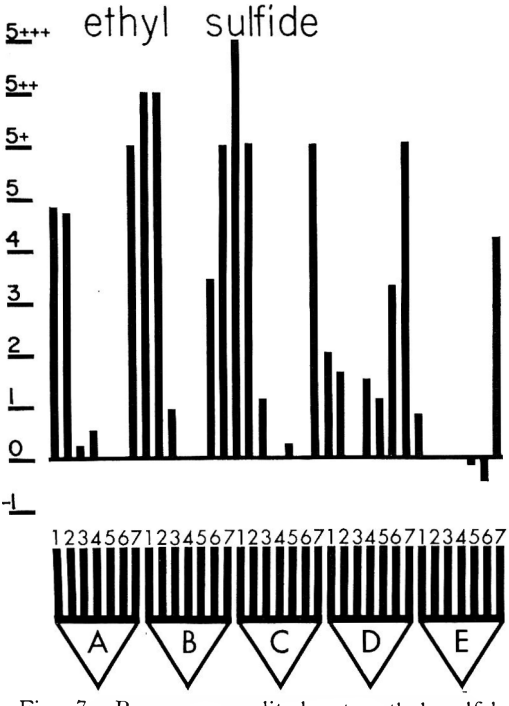

Fig. 7. Response amplitudes to ethyl sulfide under 35 electrode conditions with a 0.005-inch-diameter Pt microelectrode. Electrolytes: *A*, 0.2 *M* KH_2PO_4 + 0.3*M* Na_2HPO_4 + 0.2*M* NaCl; *B*, 0.1*M* Na_2CO_3 + 0.1*M* $NaHCO_3$; *C*, 0.1*M* citric acid + 0.1*M* Na citrate; *D*, deionized water with less than 0.2 ppm NaCl equivalent conductivity; *E*, 0.1*M* glycine. Applied voltage: 1, −1.5 volts; 2, −1.0 volt; 3, −0.5 volt; 4, 0.00 volt; 5, +0.5 volt; 6, +1.0 volt; 7, +1.5 volts. A 1-cc sample of saturated odorant headspace at 75°F was used with an airflow velocity of 440 cm/min. Response amplitudes shown are 5 × 10^{-8} amps (5 = 25 × 10^{-8} amps).

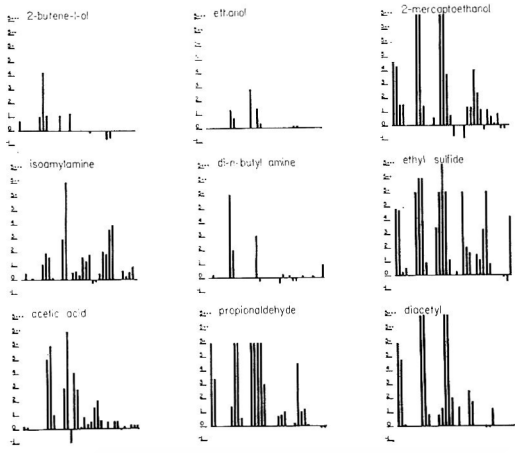

Fig. 8. Response amplitude profiles with 35 electrode conditions. Electrode conditions are identical to those shown in Fig. 7.

electrode surface or, in other words, to a saturation condition. This plot of stimulus intensity vs. response shows a power function relationship which conforms with psychophysical experiments. For some odorants, sensitivities were as high as 3 × 10^{-14} moles/cc/sec.

Amplitude responses to the volatiles of three onion varieties (Fig. 11) illustrate some of the results that may be obtained with a single electrode system. Since the sample preparation was sealed (Waring blender) the curves indicated a possible rapid decomposition of some volatile, probably the lachrymogenic compound thiopropional-S-oxide (Wilkens, 1961). Propional, the major constituent of onion volatiles, was added to one variety at the end of 15 min of blending to make sure leakage in the system was not involved. Continued blending and sampling showed only a slight decrease in response amplitude with time. Different profiles obtained with other foodstuffs are not presented at this time. Some of the materials tested that gave responses at one or more electrode conditions were: CO, SO_2, HCN, potato chips, peas, butter, cheese, Glade mist air freshener, ethyl iodide, Parathion, amino triazole, and Eptam.

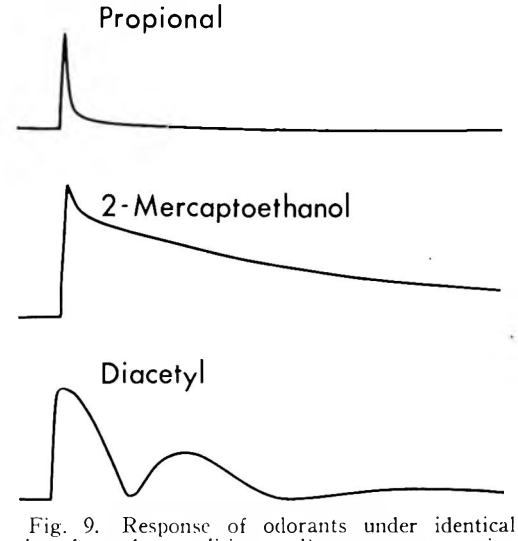

Fig. 9. Response of odorants under identical microelectrode conditions. Response attenuation ratio differs for each odorant. Electrolyte, 0.1*M* $NaHCO_3$ + 0.1*M* Na_2CO_3; potential, −1.0 volt; microelectrode, 0.005-inch-diameter Pb wire; macroelectrode, Pt; odorant sample, 1 cc saturated headspace at 75°F; airflow velocity, 440 cm/min.

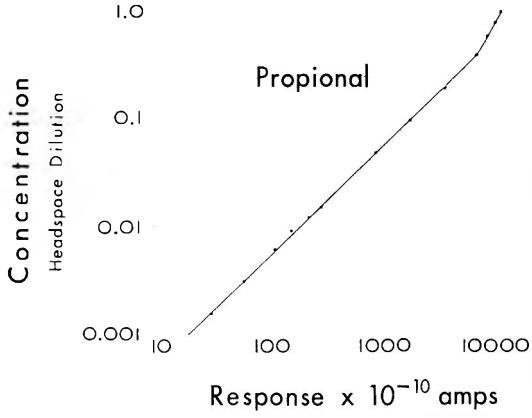

Fig. 10. Response amplitude as a function of odorant concentration. Concentration of 1.0 = saturated headspace at 75°F.

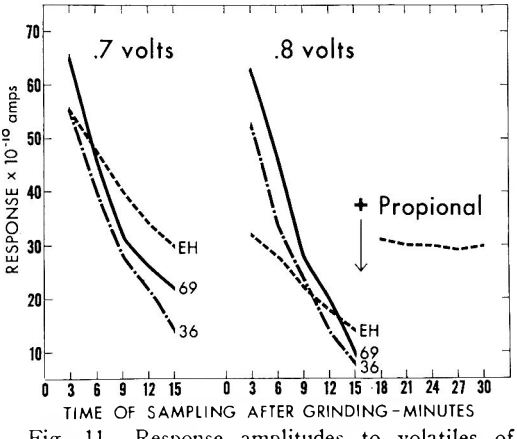

Fig. 11. Response amplitudes to volatiles of three onion varieties at a typical electrode condition. Samples were taken at 3-min intervals after initiation of blending.

A complex instrument for the nearly simultaneous measurement of odorant response at 8 different microelectrodes is presently almost complete. While we cannot learn, without a great deal of testing of this instrument and further work with such components as have been described, whether the human olfactory system actually is closely analogous to the electronic system with which the writers have been experimenting, all evidence so far seems in favor of the idea that the two systems do work similarly.

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