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CONTENTS

Anthocyanins. IV. Anthocyanin Pigments of Cabernet Sauvignon Grapes[a,b]

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(Manuscript resubmitted May 29, 1963)

SUMMARY

Six anthocyanins were isolated from Cabernet Sauvignon grapes. Four major and one minor pigment were identified by paper chromatography, color reactions, and spectroscopy. The four major anthocyanins were identified as delphinidin-3-monoglucoside, petunidin-3-monoglucoside, malvidin-3-monoglucoside, and malvidin-3-monoglucoside acetylated with chlorogenic acid. Malvidin-3-monoglucoside was the most abundant pigment of the grapes. One of the minor pigments was identified as petunidin.

INTRODUCTION

In previous investigations in this laboratory, Pratt *et al.* (1960), Powers *et al.* (1960), Hamdy *et al.* (1961), and Somaatmadja (1962) found that anthocyanins influence the growth of certain bacteria. Efforts to establish the mode of action required identification of the anthocyanin pigments, especially their state of glycosidation. This paper deals with identification of the major pigments of Cabernet Sauvignon grapes.

REVIEW OF LITERATURE

Bockian *et al.* (1955) reported that Cabernet Sauvignon grapes contain delphinidin-3,5-diglucoside, petunidin-3,5-diglucoside, malvidin-3,5-diglucoside, malvidin-3-monoglucoside, malvidin, and malic acid. The number of anthocyanin pigments in various varieties was investigated by Rankine *et al.* (1958). Those investigators reported that Cabernet Sauvignon grapes contained 8 anthocyanin compounds, but the identity of the compounds was not reported. Ribereau-Gayon (1959) reviewed his own work and that of others. He concluded that *Vitis vinifera* grapes do not contain diglycosidic anthocyanins.

EXPERIMENTAL PROCEDURE

Isolation of anthocyanins. The Cabernet Sauvignon grapes were shipped by air to this laboratory from Davis, California. Some of the grapes were then blanched and frozen, and others were stored without blanching. The frozen grapes were stored at −35°C until analyzed.

The grapes were macerated in a Waring blender, and the anthocyanins were extracted twice with cold 1% hydrochloric acid solution. The combined extract was filtered through filter paper, and the anthocyanins were precipitated with lead acetate as reported by Bockian *et al.* (1955). The precipitate was washed several times with water to remove traces of free sugar.

The anthocyanins were re-extracted from the lead salt with methanol–2% hydrochloric acid solution. The $PbCl_2$ precipitate was removed by centrifugation, and the methanol-pigment extract was concentrated *in vacuo*.

The various anthocyanins were isolated by descending paper chromatographic technique using the upper phase of a BAW solvent system. The various paper chromatographic solvent systems used are listed in Table 1. The relative intensities of the different bands were determined with a photodensitometer. The chromatograms were sprayed with p-anisidine reagent to check for the possible contamination of various anthocyanins by free sugar (fructose and glucose). The chromatograms were also sprayed with phosphomolybdic acid solution to determine the presence of anthocyanins with ortho-dihydroxyl groups which produce a blue color with this reagent (Riley, 1950; Bentley, 1960).

Identification of anthocyanins. The separated anthocyanins were eluted with methanol–0.01% HCl solution. The continuous absorption spectra of the various anthocyanins were determined with a self-recording spectrophotometer. The ratios of

[a] Contribution from the College Experiment Station, University of Georgia, Athens, Georgia. Approved as Journal paper No. 186. Portions of the data were taken from the Ph.D. dissertation of Dardjo Somaatmadja, University of Georgia, 1962.

[b] This study was supported by the U. S. Public Health Service Grant, No. E-3156, and by California Wine Advisory Board Contract M-40.

Table 1. The solvent systems (components and code) used for the paper chromatographic identification of anthocyanin pigments.

Code	Components
BAW	1-butanol–acetic acid–water (4:1:5 v/v upper phase)
BEW	1-butanol–ethanol–water (10:1:2 v/v)
BPyW	1-butanol–pyridine–water (45:25:40 v/v)
Bu-HCl	1-butanol–2*N* hydrochloric acid (1:1 v/v upper phase)
EAc-Py-W	ethyl acetate–pyridine–water (2:1:2 v/v)
Forestal	acetic acid–12*N* hydrochloric acid–water (30:3:10 v/v)
HAc-HCl	acetic acid–12*N* hydrochloric acid–water (15:3:82 v/v)

the absorbancies at 440 mμ to that at which the maximum absorption occurs (E_{440}/E_{max}) were calculated to determine the state of glycosidation of the pigments (Harborne, 1958b).

The ascending R_f values of the separated pigments were determined with the BAW, Forestal, Bu-HCl, and HAc-HCl solvent systems (see Table 1).

Identification of anthocyanidin and sugar moieties. A 1:1 mixture of anthocyanins and 2*N* HCl solutions was placed in a boiling-water bath for 30 min. The reaction mixture was cooled, and the anthocyanidin fractions were extracted with n-amyl alcohol. The aqueous phase was saved for sugar determinations.

The anthocyanidins were chromatographed ascendingly with the Forestal, Bu-HCl, and HAc-HCl solvents. Synthetic delphinidin, petunidin, and malvidin were also chromatographed with the same solvent systems in order to compare the characteristics of the natural anthocyanidins with those of the synthetic aglycones.

The aqueous phase containing sugar fractions of the pigments was neutralized with NaOH solution. The sugar moieties were determined by paper chromatography with the BEW, BPyW and BAW solvents (Table 1).

Identification of acylated anthocyanins. The anthocyanins were saponified in a 1% HCl solution with 50% NaOH solution added to yield a concentration of 2*N* NaOH, and the reaction mixtures were held 5 min at room temperature. The mixtures were acidified with HCl, and the liberated acids were extracted with diethyl ether. The ether fractions were chromatographed using the BEW, BPyW, and EAc-Py-W solvents (Table 1). The chromatograms were sprayed with chlorophenol red indicator.

RESULTS AND DISCUSSION

Six well-defined bands were observed when pigment extract of Cabernet Sauvignon grapes was paper-chromatographed descendingly using the upper phase of the BAW solvent system. The descending R_f values and other characteristics of the various bands are shown in Table 2. Ascending R_f values of these bands in various solvent systems are shown in Table 3. R_f values obtained by these two methods differed slightly. This fact has been reported by Harborne (1958a) and Pruthi *et al.* (1961).

The relative intensities of the various bands, measured photodensitometrically, are shown in Fig. 1. Bands 1, 2, 3, and 5 represent the major pigments of this grape variety. Band 0 represents the fraction of pigments that did not move on the paper

Table 2. Descending R_f values and other characteristics of anthocyanin pigments isolated from Cabernet Sauvignon grapes.

Band	R_f in BAW (upper)	Color characteristics			Relative intensity
		Visible	UV light	Phosphomolybdic acid	
0	.....	brown	brown	brown	moderate
1	0.15	light blue	bluish purple	faint blue	moderate
2	0.20	light blue	bluish purple	blue	moderate
3	0.30	pink	lavender	fuchsia	large
4	0.38	pink	.....	faint blue	trace
5	0.41	pink	pink	pink	moderate
6	0.49	pink	.....	faint blue	small amount
7	0.67	.....	blue fluor	.....	.....

Table 3. Ascending R_f values and absorption characteristics of anthocyanin pigments isolated from Cabernet Sauvignon grapes.

Band no.	R_f values in				Absorption characteristics	
	BAW (upper)	Forestal	Bu-HCl	HAc-HCl	λ_{max} (mμ)	% E_{440}/E_{max}
1	0.18	0.50	0.13	0.20	554	26
2	0.24	0.60	0.18	0.25	546	23
3	0.31	0.78	0.21	0.32	540	22
4	0.32	0.80	0.18	.....	.....	.....
5	0.44	0.80	0.20	0.25	540	24
			0.38			
6	0.49	0.82	0.45	0.10	.....	.....
malvidin-3,5-diglucoside	0.25	0.85	0.08	0.51	536	11

chromatogram using the upper phase of the BAW solvent. Bands 1, 2, 4, and 6 produced a blue coloration when sprayed with phosphomolybdic acid solution, indicating the presence of ortho-dihydroxy anthocyanins (Riley, 1950; Bentley, 1960).

Investigations on band 1 show that the R_f values of this band in four different solvent systems (Table 3) agreed very closely with values reported by Bate-Smith (1950), Abe and Hayashi (1956), and Harborne (1958a) for delphinidin-3-monoglucoside. Acid hydrolysis of this pigment gave anthocyanidin with R_f values in three solvent systems (Table 4) similar to those of synthetic delphinidin. The blue coloration with phosphomolybdic acid shows that this pigment has *O*-dihydroxyl group as in delphinidin. Glucose was the only sugar obtained by acid hydrolysis of this anthocyanin. The R_f values of the sugar fraction of this pigment in three solvent systems are shown in Table 5. Negative Seliwanoff and Tollen's tests respectively eliminated the presence of fructose and galactose (Hawk and Bergeim, 1937).

The maximum absorption peak of this pigment was at 546 mμ (λ_{max} = 546 mμ),

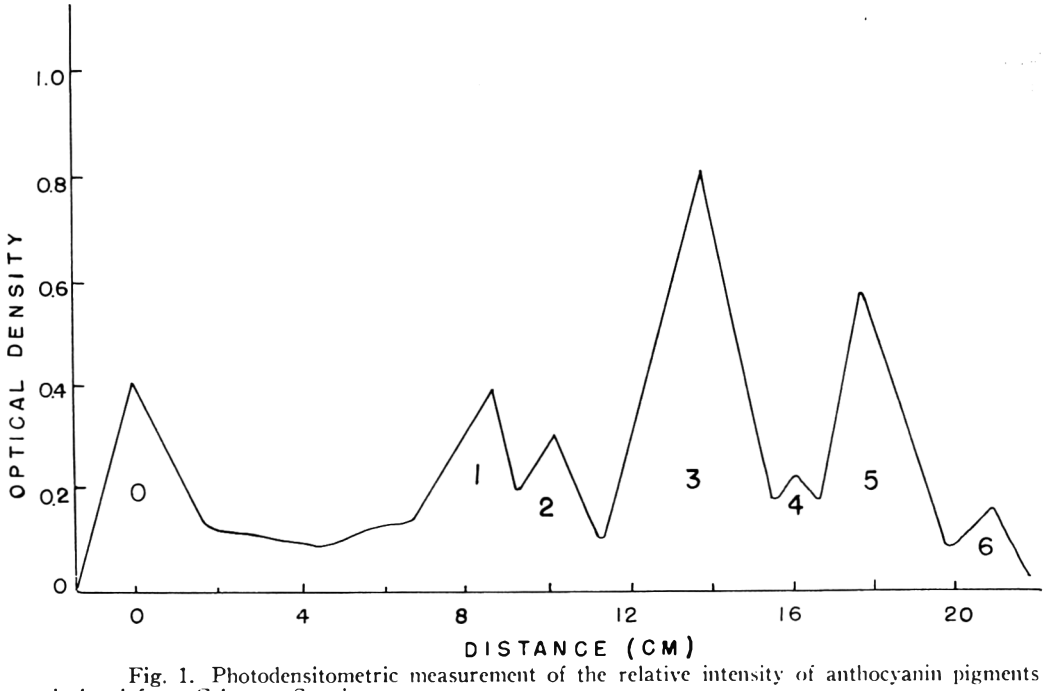

Fig. 1. Photodensitometric measurement of the relative intensity of anthocyanin pigments isolated from Cabernet Sauvignon grapes.

Table 4. Identification of aglycones obtained by acid hydrolysis of anthocyanin pigments of Cabernet Sauvignon grapes.

Band no.	R_f values in			Color reaction with phosphomolybdic acid	Identification
	Forestal	Bu-HCl	HAc-HCl		
1	0.36	0.37	0.06	blue	delphinidin
2	0.50	0.46	0.08	faint blue	petunidin
3	0.64	0.53	0.13	pink	malvidin
4					
5	0.63	0.50		pink	malvidin
6	0.49	0.46		faint blue	petunidin
delphinidin *	0.35	0.37	0.06	blue	
petunidin *	0.50	0.44	0.08	faint blue	
malvidin *	0.64	0.52	0.12	pink	

* Synthetic samples.

Table 5. Identification of sugar fraction or anthocyanin pigment on Cabernet Sauvignon grapes.

Band no.	Qualitative tests			R_f values in			Identification
	Benedict	Seliwanoff	Tollens	BAW	BPyW	BEW	
1	+	—	—	0.21	0.40	0.13	glucose
2	+	—	—	0.20	0.39	0.12	glucose
3	+	—	—	0.21	0.41	0.12	glucose
5	+	—	—	0.21	0.40	0.12	glucose
6	—	—	—				
glucose	+	—	—	0.21	0.40	0.13	
fructose	+	+	—	0.25	0.46	0.17	

and the E_{440}/E_{max} value was 26%. These values did not agree with those reported by Harborne (1958b). He found that the λ_{max} and E_{440}/E_{max} values for 3-monoglucosides of delphinidin, petunidin, and malvidin were 534 mμ and 18%. Apparently the absorption characteristics cannot be used to differentiate among the three anthocyanins above. This characteristic is useful, however, to differentiate among aglycones and mono- and diglycosides.

Based on the R_f values, sugar analysis, and characterization of the anthocyanidin, band 1 was identified as delphinidin-3-monoglucoside. Bockian *et al.* (1955) reported that the pigment of Cabernet Sauvignon grapes with the lowest R_f value ($R_f = 0.11$) was delphinidin-3,5-diglucoside. However, they did not use synthetic delphinidin-3,5-diglucoside for comparison.

Acid hydrolysis of the pigment of band 2 gave anthocyanidin with R_f values of 0.50, 0.46, and 0.08 in the Forestal, Bu-HCl, and HAc-HCl, respectively (Table 4). Synthetic petunidin gave R_f values of 0.50, 0.44, and 0.08, respectively, in the above solvent systems. It seems that the anthocyanidin part of this pigment is petunidin. Before and after acid hydrolysis this pigment produced a faint blue coloration with phosphomolybdic acid solution, indicating the presence of *O*-dihydroxyl group as in petunidin. The difference of the intensity of the blue coloration of band 1 and band 2 may be accounted for by the fact that delphinidin has three OH groups on the side ring and petunidin has only two.

Sugar analysis of this pigment gave only glucose (Table 5). No fructose or galactose was observed, as indicated by negative Seliwanoff and Tollen's tests.

Before acid hydrolysis, pigment of band 2 had R_f values in the Bu-HCl and HAc-HCl of 0.18 and 0.25, respectively. These values are in general agreement with those reported by Harborne (1958a) for petunidin-3-monoglucoside. Bockian *et al.* (1955) reported that this band was petunidin-3,5-diglucoside. They found an R_f value in the BAW system of 0.15. The R_f value for petunidin digluco-

side reported by Harborne (1958a) in the same solvent system was 0.24. In this study the R_f value of this band was 0.24. However, should the pigment found in this study be petunidin-3,5-diglucoside (same R_f value as found by Harborne) the R_f values in Bu-HCl and HAc-HCl should be 0.04 and 0.32, respectively, which was not the case. Bockian *et al.* (1955) used only one solvent system. Based on the above comparison, band 2 was identified as petunidin-3-monoglucoside. The discrepancy in the R_f value in BAW solvent found in this study and that found by Harborne may be due to the difference in the age of the solvent. Here, freshly prepared solvent was used; Harborne employed 3-day-old BAW solvent.

The characteristics of the aglucone obtained by acid hydrolysis of pigment 3 were similar to those of synthetic malvidin (Table 4). Glucose was the only sugar found in this pigment. Investigations on R_f values of this pigment before acid hydrolysis in different solvent systems indicate that this pigment is malvidin-3-monoglucoside. The R_f values in Bu-HCl and HAc-HCl of this pigment are in general agreement with those reported by Harborne (1958a).

A comparison of R_f values and absorption characteristics of this pigment with those of synthetic malvidin-3,5-diglucoside (malvin) are shown in Table 3. None of the characteristics of pigment 3 matched those of malvin. The E_{max} and E_{440}/E_{max} values were higher than those of malvin, which gives further support to the identity of this band as malvidin-3-monoglucoside instead of malvin. Glycosidation shifts the λ_{max} to shorter wavelength (Ribereau-Gayon, 1959) and decreases the E_{440}/E_{max} value (Harborne, 1958b).

The pigment of band 4 was so minute that extensive chemical tests were not made. In several chromatograms this band could not be detected.

Band 5 gave two R_f values in the Bu-HCl solvent (Table 3). The lower R_f value was similar to that of band 3, which was identified as malvidin-3-monoglucoside. Acid hydrolysis of band 5 gave anthocyanidin with R_f values in the Forestal, Bu-HCl, and HAc-HCl solvents similar to those of synthetic malvidin (Table 4). Glucose was also found in this pigment, as shown in Table 5.

Table 6. R_f values of some organic acid.

Acid	R_f values in		
	BEW	B-Py-W	EAc-Py-W
caffeic	0.73	0.72	0.82
chlorogenic	0.50	0.59	0.63
vanillic	0.69	0.63	0.70
gallic	0.55	0.60	0.70
coumaric	0.82	0.69	0.86
acid from band 5	0.49	0.60	0.64

Upon saponification the pigment yielded an acid with R_f values in three different solvents similar to those of chlorogenic acid. The spot of this acid on paper produced a green coloration with ammonium hydroxide fumes, as does chlorogenic acid. The appearance under UV light, with and without ammonia fumes, was similar to that of chlorogenic acid. Harborne (1958a) reported that acylated anthocyanins give two R_f values in the Bu-HCl solvent. He listed R_f values of several acylated anthocyanins, and it appears that R_f values of acylated anthocyanins in the BAW solvent were higher than those of non-acylated anthocyanins.

Band 6 produced a faint blue coloration with phosphomolybdic acid solution, indicating the presence of *O*-dihydroxy anthocyanin. R_f values of this pigment before and after acid hydrolysis were similar to those of petunidin (Tables 3 and 4). Negative Benedict's, Seliwanoff, and Tollen's tests were observed on hydrolysate of this pigment, indicating that it is an aglycone. Based on chromatographic properties of this pigment it was identified as petunidin.

Band 7 (Table 2) did not show any color, but it fluoresced under UV light. With ammonia fumes this band gave a light-green color, a characteristic similar to that of chlorogenic acid. The appearance under UV light with and without ammonium hydroxide fumes was similar to that of chlorogenic acid.

The above results indicate difficulties in identification studies of anthocyanin pigments based on R_f values alone. Unless conditions such as the acidity of samples before being applied onto the paper (Bate-Smith,

1950), solvent system (Harborne, 1958a), and technique (Harborne, 1958a; Pruthi *et al.*, 1961) be exactly the same, the R_f value of an anthocyanin cannot be used as the only criterion for comparison with values reported in the literature. The above factors have been reported by those investigators to influence R_f values. To avoid such difficulties, synthetic samples with known identity should be used for comparison.

The results of this study agreed partially with those reported by Bockian *et al.* (1955) in that glycosides of delphinidin, petunidin, and malvidin were found. However, the state of glucosidation of the compounds found in this study was not the same as those reported by Bockian *et al.* (1955). Those investigators reported that the most abundant pigment was malvidin-3,5-diglucoside, based on the use of one solvent. In this study, however, malvidin-3-monoglucoside was found to be the most abundant pigment. Identification was based on the fact that the R_f values and other characteristics under the same conditions of test did not match those of synthetic malvidin-3,5-diglucoside.

The presence of diglycosidic anthocyanins in *Vitis vinifera* grapes has been a subject of controversy. Willstaetter and Zolinger (1916) reported that *V. vinifera* contained malvidin diglucoside. However, Ribereau-Gayon (1959) concluded, based on his own work and that of others, that *vinifera* grapes do not contain diglycosidic anthocyanins.

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The Carotenoids of Italian Prunes

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SUMMARY

The major constituents of the carotenoid mixture of Italian prunes were found to be violaxanthin (35%), beta-carotene (19%), and lutein (16%). Less abundant constituents included cryptoxanthin, cryptoxanthin-5,6-epoxide, cryptoxanthin 5,6,5′,6′-diepoxide, persicaxanthin, luteoxanthins, antheraxanthin, phytoene, and phytofluene. About 20 other carotenoids were found as very minor constituents. The carotenoid mixture in Italian prunes was notable for its low content of 5,8-epoxides.

In recent years the carotenoids of a number of fruits have been investigated at this laboratory, including Valencia oranges (Curl, 1953; Curl and Bailey, 1954), cling peaches (Curl, 1959), apricots (Curl, 1960a), Japanese persimmons (Curl, 1960b), and tomatoes (Curl, 1961). This study has now been extended to Italian prunes. Except for peaches and apricots, very little has been published on the carotenoids of any fruits of the genus *Prunus*. Italian prunes (a variety of *Prunus domestica*) are a good source of beta-carotene (Strachan *et al.*, 1951).

EXPERIMENTAL

Italian prunes originating in Idaho were obtained at a local market in early October. Fruits selected were ripe but rather firm, deep purple outside, and greenish to brownish inside. The quantity of deseeded fruit used was 2100 g.

Extraction. The fruit, in 250-g batches, was blended with 250 ml of water and 5.8 g of magnesium carbonate, then added to 250 ml of methanol. After a few minutes, Celite 503 (10% by weight of the fruit used) was added, and the mixture was worked up essentially as previously described (Curl, 1961; Curl and Bailey, 1959), including saponification.

Countercurrent distribution. The saponified carotenoids were separated into (a) hydrocarbons, (b) monols, and (c) diols and polyols by means of a 100-transfer countercurrent distribution run in a Craig apparatus with solvent system I (Curl, 1953). The fractional composition of the diol-polyol fraction was determined by means of a 100-transfer run, using a small aliquot, with solvent system IV (Curl, 1960c). The diol-polyol fraction was then separated into (a) diols, (b) monoepoxide diols, (c) diepoxide diols, and (d) polyols by means of a 208-transfer run with system II (Curl, 1953). The N_{100} values of several polyol constituents were determined in solvent system III (Curl, 1960c).

Chromatography. The hydrocarbon and monol fractions obtained by distribution with solvent system I, and the diol, monoepoxide diol, diepoxide diol, and polyol fractions obtained with system II, were each chromatographed on columns of magnesia (Sea Sorb 43), 14 by *ca.* 90 mm, using a graded series of eluants (Curl and Bailey, 1959). Spectral data were obtained with a Beckman model DK-2 recording spectrophotometer.

RESULTS AND DISCUSSION

The total carotenoid content (after saponification) of the fruit was found to be equivalent to 21 mg/kg (as beta-carotene), a fairly high content.

Countercurrent distributions. The fractional composition of the carotenoids is given in Table 1. The distribution curve for the monol fraction (System I) was asymmetrical, indicating the presence of lesser amounts of monol epoxides, which have lower N_{100} values than cryptoxanthin; about one-third of the monol fraction apparently consisted of monol epoxides.

Chromatography. Thirty constituents obtained on chromatography of the various fractions are given in Table 2; 12 amounted to 1% or more of the carotenoid mixture. Within each fraction, the constituents are listed in order of elution.

Hydrocarbons. Beta-carotene was the predominant component of this fraction. Phytoene, phytofluene, and zeta-carotene

Table 1. Fractional composition of the carotenoids of Italian prunes as determined by countercurrent distribution with solvent systems I and IV.

Fraction	Percent	N_{100}[a]
System I		
I (Hydrocarbons)	23	91
II (Monols)	9[b]	57
III (Diols and Polyols)	67	6
System IV		
IIIA (Diols)	22	82
IIIB (Monoepoxide diols)	2	50
IIIC (Diepoxide diols)	37	19
IV (Polyols)	6	1

[a] Position of maximum per 100 transfers.
[b] Estimated, from shape of distribution curve, to contain about one-third monol epoxides.

were present in relatively small quantities; one minor fraction, tentatively identified as mutatochrome (Table 3), was present in an amount too small for further investigation.

Monols. The principal component of the monol fraction was cryptoxanthin, a common major constituent of fruit carotenoids, and a provitamin A. Below it on the column were two bands with similar spectral absorption curves resembling that of hydroxy-alpha-carotene. The lower, lesser band was apparently hydroxy-alpha-carotene. The spectral absorption maxima of an aliquot of the upper band in methanol shifted from 473, 445, and 420 (shoulder) to 452, 426, and 402 mμ on addition of a drop of concentrated hydrochloric acid, indicating the presence of one 5,6-epoxide group as in cryptoxanthin 5,6-epoxide.

The remainder of the upper band was used in a countercurrent distribution run with solvent system I. The main maximum had an N_{100} of 48, in agreement with that of cryptoxanthin 5,6-(or 5′,6′-) epoxide, previously obtained from Meyer lemon peel (Curl, 1962). There was also a definite shoulder at about tube 34, in good agreement with the value for cryptoxanthin 5,6,5′,6′-diepoxide (32-3) (Curl, 1962). Spectral absorption data measured before and after addition of hydrochloric acid confirmed these identifications.

P 374 (Table 3) did not occur as a visible band on the column, but the spectral absorption maxima were observed in the fractions that also contained substances tentatively identified as cryptoxanthin 5,6,5′8′-diepoxide and cryptoxanthin 5,8-epoxide. Similar substances had previously been found in Navel orange peel (Curl and Bailey, 1961) and tomatoes (Curl, 1961). P 374 apparently has a system of six conjugated double bonds, but little further is known about it.

Diols. The diol fraction consisted almost entirely of lutein, with a much smaller amount of zeaxanthin; in some other fruits, zeaxanthin is the greater.

Table 2. Carotenoid constituents of Italian prunes.

Fraction[a]	Constituent	Approximate % of carotenoid mixture[b]
I	Phytoene	1.3
	Phytofluene	1.1
	alpha-Carotene	0.5
	beta-Carotene	18.7
	zeta-Carotene	0.2
	Mutatochrome[c]	0.4
II	Hydroxy-alpha-carotene	0.2
	Cryptoxanthin 5,6,5′,6′-diepoxide	1.2
	Cryptoxanthin 5,6-epoxide[c]	3.5
	Cryptoxanthin	7.3
	Cryptoxanthin 5,6,5′,8′-diepoxide[c]	0.4
	Cryptoxanthin 5,8-epoxide	0.6
	P 374	1.2
IIIA	Lutein	15.5
	Zeaxanthin	0.4
IIIB	Lutein 5,6-epoxide	0.4
	Antheraxanthin	2.1
	Flavoxanthin	0.04
	Mutatoxanthins	0.2
IIIC	Carbonyl *a*	0.2
	Carbonyl *b*	0.2
	Violaxanthin	35.0
	Luteoxanthins	2.3
IV	Persicaxanthin	2.9
	Neoxanthin *a*	0.4
	Neoxanthin *b*	0.4
	Trolliflor[c]	0.6
	Taraxanthin[c]	0.07
	P 399	0.3
	Persicachromes	0.1

[a] See Table 2 for explanation of I, II, IIIA, etc.
[b] Percentages based upon total absorbance of each constituent at its principal spectral absorption maximum.
[c] Tentative identification.

Table 3. Spectral absorption maxima of some carotenoids obtained from Italian prunes.

Component	Solvent [a]	Absorption maxima (mμ)
Mutatochrome [b]	H	451, 426, (401) [c]
Cryptoxanthin 5,6,5′,6′-diepoxide	H	469, 438, 414
Cryptoxanthin 5,6-epoxide	H	472, 443, (419)
Cryptoxanthin 5,6,5′,8′-diepoxide	H	448, 420
Cryptoxanthin 5,8-epoxide	H	452, 424
P 374	H	396, 374, 356
Carbonyl *a*	B	(437), 412, (388)
Carbonyl *a*	E	410
Carbonyl *b*	B	(462), 434, (408)
Carbonyl *b*	E	432
Neoxanthin *a*	B	480, 447, (421)
Neoxanthin *b*	B	479, 447, 423
Trolliflor [b]	B	479, 446, 422
cis-Trolliflor [b]	B	476, 444, 422
P 399	B	426, 399, 375

[a] H, hexane; B, benzene; E, ethanol.

[b] Tentative identification.

[c] Values in parentheses represent shoulders or inflections on spectral absorption curves, not maxima.

Monoepoxide diols. The minor monoepoxide diol fraction contained the 5,6-epoxides of zeaxanthin (antheraxanthin) and lutein, together with much smaller amounts of the corresponding 5,8-epoxides.

Diepoxide diols. The main constituent of the diepoxide diol fraction, and the most abundant of all of the carotenoids in Italian prunes, was the 5,6,5′,6′-diepoxide of zeaxanthin, violaxanthin. The 5,6,5′,8′-diepoxide isomers of violaxanthin (luteoxanthins) were found in much smaller amount. Also occurring in the diepoxide diol fraction were two minor components; comparison of the spectral absorption curves of these in benzene and in ethanol (carbonyls *a* and *b*, Table 3) indicated that both contained a carbonyl group as a part of the conjugated double bond system.

Polyols. The chromatography of the polyol fraction was much more clean-cut than is usual for fruit carotenoids. The lowest significant band was pale in color and had an orange fluorescence in ultraviolet light. It was identified as persicaxanthin (Curl, 1959) rather than valenciaxanthin (Curl and Bailey, 1954) by the N_{100} value in system III of 41, and by the strong red-violet color in the hydrochloric acid-ether test. This was by far the most abundant in the polyol fraction.

Above the persicaxanthin band on the column was a series of 4 distinct orange bands. Above these the color was considerably paler, with no apparent bands in visible light; in ultraviolet light there were two bands with greenish fluorescence (persicachromes *a* and *b*, the 5,8-epoxide isomers of persicaxanthin). The spectral absorption maxima of the 4 orange bands were similar (Table 3), except that those of the last one were at somewhat shorter wavelength, corresponding to a *cis*-isomer. The N_{100} values of these 4 fractions in system III were 63, 62, 31, and 32. The first two bands, which were identified as neoxanthins *a* and *b* (Curl and Bailey, 1957), occurred in approximately equal amounts. The principal component of the 3rd band was apparently identical with a substance previously obtained from peaches and Valencia oranges (Curl, 1960c), which was tentatively identified as trolliflor, a 5,6-epoxide that appears to be a tetraol (Lippert and Karrer, 1956). The 4th band was tentatively identified as a *cis*-trolliflor. Fractions 3 and 4 had a minor component with an N_{100} value of 50–51, in good agreement with that of a polyol previously obtained from cling peaches (Curl, 1960c), which was tentatively identified as taraxanthin. Taraxanthin is also a 5,6-epoxide, which is appar-

ently a triol-like neoxanthin (Eugster and Karrer, 1957). The difference in N_{100} value indicates that the third hydroxyl group may be on a different carbon atom from that in the neoxanthins.

The fractions eluted from the column above the 4 orange bands were mixtures containing small amounts of trolliflor-like substances and the persicachromes. One component (P 399) (Tables 2 and 3) had spectral absorption maxima similar to those of sinensiaxanthin, a 5,6-epoxide polyol of incompletely known structure obtained from Valencia oranges (Curl and Bailey, 1954). The substance obtained in the present work was eluted much more slowly than sinensiaxanthin, and had an N_{100} value in system III of *ca.* 5, whereas the value of sinensiaxanthin in this system was 46 (Curl, 1960c). The substance in the present work apparently contained more hydroxyl groups.

The percentages of all the 5,6-epoxides greatly exceeded those of the corresponding 5,8-epoxides. In apricots (Curl, 1960a) and soft ripe Elberta peaches (Curl, 1959), there were relatively much larger amounts of 5,8-epoxides. Prunes are a fairly acid fruit, and 5,6-epoxides are rapidly converted into 5,8-epoxides on contact with acids. In the prunes, the firm texture may have prevented contact between the acid and the carotenoids.

NOTICE

Reference to a company or product name does not imply approval or recommendation of the product over others.

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Determination of Malonaldehyde by Ultraviolet Spectrophotometry [a]

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SUMMARY

Malonaldehyde occurs mainly as the enol form (CHOH = CHCHO) in aqueous solution. The UV absorption spectrum of the compound is pH-dependent. Below pH 3.0, the compound is *s*-cis, planar, having an intramolecular H bond, with absorption maximum at 245 mμ and molar absorptivity (ϵ) = 1.34×10^4. Above pH 7.0, the compound is completely dissociated and the maximum absorption of the enolate anion occurs at 267 mμ with $\epsilon = 3.18 \times 10^4$. The absorbance difference between acidified and basified malonaldehyde solutions at 267 mμ can be used as a measure of malonaldehyde even in the presence of other compounds that absorb in this spectral region, provided their absorption is not pH-dependent. This difference is directly proportional to malonaldehyde concentrations from 5×10^{-6} *M* to 3×10^{-5} *M*. The method has been successfully applied to the assay of malonaldehyde in distillates from rancid foods. Its sensitivity is only about 40% of the 2-thiobarbituric acid (TBA) test, but is sufficient to detect threshold levels of rancidity. The test is simpler, much more rapid, and more specific than the TBA test.

The production of malonaldehyde has been widely used as a measure of the oxidation of unsaturated fatty acids in foods and animal tissues. It is a primary oxidation product of fatty acids having 3 or more double bonds (Dahle *et al.*, 1962). In complex foods containing peroxide decomposers such as trace metals and heme compounds, production of malonaldehyde by secondary oxidations of unsaturated aldehydes may be expected at an early stage in the oxidation of all unsaturated fatty acids. Whatever the mechanism of its production, malonaldehyde has proved extremely useful as an indicator both of flavor deterioration in many foods and of the effectiveness of antioxidants in foods and biological preparations (see numerous references in Schultz, 1962).

The test most widely used for the detection of this compound is based upon its reaction with 2-thiobarbituric acid to give a pink pigment (TBA test). Although quite sensitive, this test is open to the objection that the reagent itself is not stable under test conditions frequently employed, and various side reactions may occur (Tarladgis *et al.*, 1962).

During a broad investigation of the chemistry of malonaldehyde in this laboratory, attention was focused on the ultraviolet absorption of this compound as the basis of a new method for its quantitative assay. Malonaldehyde exists in aqueous solution mainly as the enolic form, β-hydroxy acrolein (CHOH = CHCHO). Mashio and Kimura (1960) published the ultraviolet absorption spectra at various pH values. The absorption is pH-dependent, shifting progressively from pH 3 to 7. This behavior is attributed to the progressive dissociation of the enolic hydrogen with increasing pH. At pH 3 or lower, the compound is believed to have a planar and *s*-cis-configuration, with an intramolecular hydrogen bond; above pH 7 it is completely dissociated and exists as a planar and *s*-trans-enolate anion. This hypothesis has been substantiated by molecular orbital calculations and quantum

[a] A report of work done under contract with the U. S. Department of Agriculture and authorized by the Research and Marketing Act of 1946. This contract is supervised by the Eastern Utilization Research and Development Division of the Agricultural Research Service.

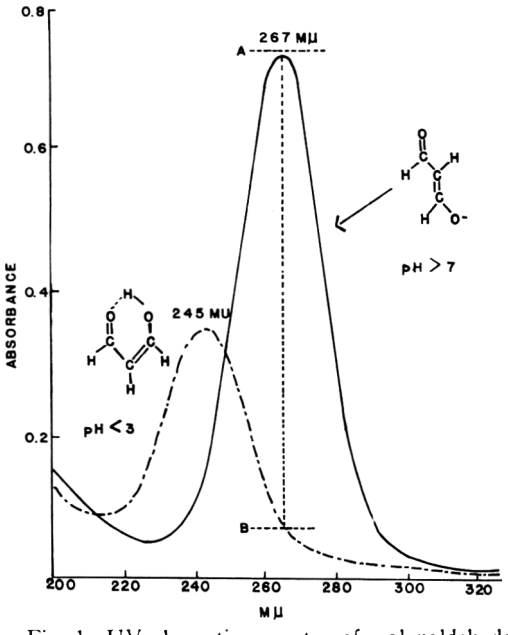

Fig. 1. UV absorption spectra of malonaldehyde in aqueous solution.

chemical interpretations of the spectra (Kwon, 1963).

Fig. 1 illustrates the structures of these two configurations of β-hydroxy acrolein and their absorption spectra. The spectra were obtained on a Bausch and Lomb Spectronic 505 recording spectrophotometer on identical concentrations of malonaldehyde adjusted to the pH values indicated. As the pH is decreased below 3, or increased above 7, no further changes in the spectra occur. At λ 267 mμ (the absorption maximum for the anion), the absorption of the acidified solution is very low. The difference between the absorptions of the acidified and the basified solutions, represented by the line AB, is therefore proposed as a quantitative measure of malonaldehyde. This paper explores the possibility of adapting this method to the assay of malonaldehyde in distillates from rancid foods.

EXPERIMENTAL

Preparation of standard malonaldehyde solutions. Malonaldehyde is not commercially available in the pure form. Malonaldehyde bis-(diethylacetal), (1,1,3,3-tetraethoxypropane) was obtained from Kay-Fries Chemicals, New York. This compound was further purified by distilling twice and collecting the portion boiling at 211.5°C, which was a colorless, clear liquid. Hydrolysis of the compound to give malonaldehyde was accomplished as follows: A weight of 0.2204 g (10^{-3} moles) was dissolved in 200 ml of double-distilled water in a 1-L Erlenmeyer flask. One ml of 1*N* HCl was added. A glass stopper was held in place firmly with stopcock grease to prevent loss of malonaldehyde while heating. After 60 min in a water bath at 50°C, the flask was cooled to room temperature, made to volume, and further diluted as necessary. Diluted solutions (concentrations below 2.5×10^{-5} *M*) with pH adjusted as indicated in Fig. 1, showed no change in absorbance after 20 days in the refrigerator. Thus the compound appears to be reasonably stable under these conditions.

Standardization. The malonaldehyde solution prepared as described above was diluted to an appropriate range for spectrophotometry. Two 10-ml portions of each concentration were adjusted to pH 11.3 and 2.25 by the addition of 0.1 ml of 1*N* NaOH and HCl, respectively. The absorbance of both acidified and basified solutions were then read against water as a blank and against each other with a Beckman DU spectrophotometer and a 1-cm cell. The results (Fig. 2) show the expected linear relation between absorbance and concentra-

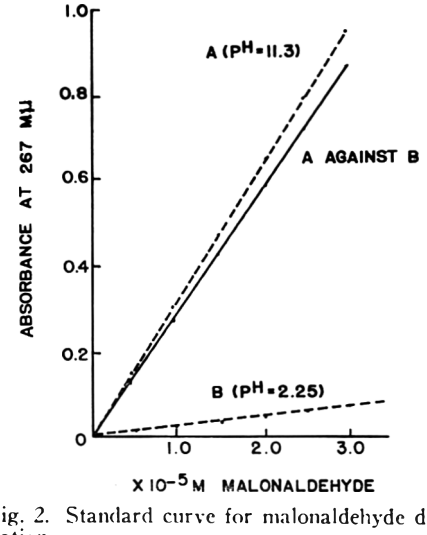

Fig. 2. Standard curve for malonaldehyde determination.

tion of both acidified and basified solutions. The line (A against B) may therefore be taken as the standard curve for the quantitative determination of malonaldehyde by ultraviolet spectrophotometry.

Aliquots of the above standard solutions were also analyzed for malonaldehyde by the TBA method (Tarladgis *et al.*, 1960). The ratio of the absorbance of the malonaldehyde TBA pigment (read at 532 mμ) to that of UV absorbance was 2.42. Molar absorptivity of the malonaldehyde TBA complex was 2.8% higher than that found by Tarladgis *et al.* (1960). This difference may be ascribed to further purification of the malonaldehyde precursor by distillation.

Noninterference of other aldehydes. Although a large number of other aldehydes are produced during the oxidation of unsaturated lipids, none of these are expected to interfere with the ultraviolet assay of malonaldehyde. Saturated aldehydes (formaldehyde, acetaldehyde, propionaldehyde, butyraldehyde, and hexaldehyde) showed absorption peaks in the range of 278 to 288 mμ. However, molar absorptivities for these $n \rightarrow \pi^*$ electronic transitions were very low (ϵ = 1 to 10) and there were no differences in the absorbances as a function of pH. The α,β-unsaturated aldehydes, acrolein and crotonaldehyde, absorb in the region of 210 to 225 mμ. Molar absorptivities (approximately 10^4) are similar to those of malonaldehyde because of strong $\pi \rightarrow \pi^*$ electronic transitions, but, again, their absorption spectra do not change with pH and therefore do not interfere in the determination of malonaldehyde.

Phenolic antioxidants. The possible interference of the most commonly used phenolic antioxidants was explored, with the following results. Because of their water insolubility, neither tocopherol nor butylated hydroxy toluene contributed to the ultraviolet absorption of aqueous distillates. Propyl gallate, when dissolved in water at concentrations of 0.001%, showed marked absorption in the ultraviolet region, and the absorption was pH-dependent. However, at the wavelength used for malonaldehyde determination (267 mμ) the absorptions of the acidified and basified propyl gallate solutions are almost identical and interference from this compound is negligible.

Distillates from butylated hydroxy anisole (BHA) emulsions or from meats containing BHA showed pH-dependent absorption in the ultraviolet region. The basified solution absorbed less at 267 mμ than the acidified solution. Thus this compound interfered drastically in malonaldehyde determinations carried out by comparing absorption of distillates at pH 11 and 2. Further exploration of the pH dependence revealed that the shift from the acid to the alkaline spectrum of BHA occurred only above pH 9.5. Since the spectrum of the enolate anion of malonaldehyde was fully developed at pH 7 or above, malonaldehyde could be determined quantitatively in the presence of BHA by adjusting the alkaline solution to pH values between 7 and 9.5 rather than to pH 11 or above.

Application of the method to foods. The ultraviolet method has been compared with the TBA method on aliquots from the same distillates of a number of food samples under investigation in this laboratory. These samples included fresh, cooked, and cured meats with various antioxidants; fish; synthetic rat rations containing a variety of added fats; and raw peas. In the analysis of cured meats, nitrite was eliminated before distillation, as suggested by Zipser and Watts (1962).

The ultraviolet spectra of distillates from most of these samples showed absorption in the ultraviolet in addition to that from malonaldehyde. In meat and fish samples, non-malonaldehyde ultraviolet absorption was minor and did not change the malonaldehyde spectra appreciably. In distillates from mixtures containing cereal products and peas, complex ultraviolet spectra were obtained, very different in appearance from those of malonaldehyde. Nevertheless, in all of these products the ultraviolet method was applicable. Ratios of TBA absorbance to ultraviolet absorbance, obtained as described under "Standardization," ranged from 2.2 to 2.6 in 30 comparisons on distillates from the foods mentioned above. The mean value was 2.4, practically identical to that obtained from pure malonaldehyde.

Except with samples containing BHA, it was not necessary to exercise care in the pH adjustment of distillates from any of the foods tested. Where BHA is present, the pH of the basified solution must be between 7 and 9.5. The concentration of added alkali needed to bring the distillate to this pH range must be determined with each class of foods, since distillates may have considerable buffering capacity.

To convert to "malonaldehyde number" (mg malonaldehyde per 1000-g sample), using a 10-g sample and assuming a recovery of 68% of the malonaldehyde from the sample in the distillate (Tarladgis *et al.*, 1960), the ultraviolet absorbance is multiplied by the factor 18.7. It should be pointed out that little is known about the relation between malonaldehyde in the distillate and malonaldehyde pre-existing in the food sample. It is quite possible that some malonaldehyde is produced from precursors during the acid distillation or that preformed malonaldehyde has reacted with other food components to give compounds that may not distill. Until further work clarifies these questions, it is probably better to designate the malonaldehyde measured in such distillates as "distillable malonaldehyde."

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Non-Enzymic Browning. Development of Chromophores in the Glucose-Glycine and Sucrose-Glycine Systems

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SUMMARY

The development of chromophores in the glucose-glycine and sucrose-glycine systems was examined. During the reaction a number of conjugated unsaturated carbonyl compounds develop, the quality present at any one time being greater as the reaction proceeds, until browning is well advanced. During this period, which follows the period of rapid pH fall and α-amino N utilization, the fluorescence of the system increases, and the quantity of water-soluble, non-volatile compounds extractable into various organic solvents increases. The pattern of extraction indicates the gradual formation of intermediates of greater dehydration and greater light-absorbing properties from smaller molecules. The presence of sulfites slowed the rate of development of conjugated unsaturated carbonylic and fluorescent compounds, and of subsequent early browning, whereas in the presence of sodium pyrophosphate, their rates of production are increased. Chromophores appear to develop at a faster rate in the presence of iron than in its absence, but apparently by a mechanism different from that occurring with phosphates.

It is suggested that the production of larger carbonylic N-containing polymers from smaller carbonyl compounds, occurs in the sugar-amino compound systems.

INTRODUCTION

In an investigation into the role of sulfur dioxide in retarding non-enzymic browning, we had reason to examine the sulfite liquor in which vegetables had been scalded (blanched), since it had been found that dehydrated vegetables which had been scalded at the beginning of a run developed browning more slowly than those scalded later. The effect may have been due to a greater earlier leaching of reactive substances, such as glucose and amino acids, though the solids content of the liquor did not usually rise above 1–2%. The alternative explanation seemed to be that the leached substances reacted in the hot scald bath and formed reactive intermediates that were absorbed by the vegetable. A specimen of potato scald liquor obtained from commercial sources contained iron at a concentration of 16 ppm and acid sodium pyrophosphate at a concentration of 150 ppm.

Chromatographic examination of the concentrated liquor extract on Florisil (100–120-mesh) gave a number of fluorescent zones. Material from these and other zones enhanced the rate of color development in glucose-glycine solutions during incubation at 40°C.

Solvent extraction of freeze-dried scald liquor solids gave a yellow fluorescent fraction, extractable with cyclohexanone, which contained a potent browning agent.

The browning of sugars with amino acids is known as the Maillard reaction. We examined (with J. P. Moody) a number of glucose- and xylose-amino acid mixtures during the course of the Maillard reaction and found that the pattern of development of fluorescent compounds depended, as expected, on the amino acid used in each case. The initial development rate of the fluorophores was much reduced when sodium metabisulfite was added to the system, presumably because of the influence of the sulfite on sugar stability.

Initial experiments showed that sulfite, added to aldose-glycine mixtures, retarded the onset of chromophore development and also reduced the quantity of conjugated unsaturated carbonyl compounds in the mixture.

Taking glucose-glycine and sucrose-glycine as our model systems, an investigation was made of the conjoint action of sulfite with added phosphate and with iron salts on the development of color, and of attend-

ant production of conjugated unsaturated carbonyl compounds.

EXPERIMENTAL

All the sugar and sugar-glycine mixtures were made up from fresh bulk solutions containing 770 g of sugar (glucose, fructose, or sucrose) in 1200 ml water. Glycine was used as a saturated solution at 25°C (approx. 25 g in 100 ml). The model solutions were made by taking 7 vols. of sugar solution and 3 vols. glycine solution or, in the absence of glycine, 3 vols. water. The additives were added in a further 2 vols. of aqueous solutions, but, where no additive was used, a further 2 vols. of water was added. The aqueous solutions of the additives were made so that the final concentrations (where added) in the model systems were: sodium pyrophosphate 10 mg/ml, ferrous sulfate 0.2 mg/ml, sodium metabisulfite 3 mg/ml (to give 2 mg SO_2/ml).

The solutions were stored in stoppered bottles at 40°C (first series, A) or at 50°C (second series, B). Carbonyl absorption was studied over the range 200–350 mμ by readings taken on an Optika recording spectrophotometer. The accompanying graphs were plotted from readings of peaks obtained in the conjugated unsaturated carbonyl region at 285 mμ.

Parallel paper chromatographic examinations were performed, using Whatman's No. 1 and No. 4 papers with isopropanol-water (4/1) as the main developing solvent, with occasional use of butanol-acetic acid-water, phenol-ammonia-water, and ethyl acetate-pyridine-water. Stains used in conjunction with paper chromatograms were alkaline silver nitrate, p-anisidine HCl, aniline hydrogen phthalate, and p-aminobenzoic-oxalic acids for sugars, and their derivatives and ninhydrin for amino-containing compounds.

The solutions to be selectively extracted with solvents were incubated in tightly stoppered glass bottles at 50°C. After periods of 3, 4, and 5 weeks of incubation the bottles were opened and the contents thoroughly extracted with 3 volumes of cyclohexanone. The cyclohexanone extract was treated first with sodium bicarbonate solution and then with sodium carbonate solution. Following this the cyclohexanone solution of neutral compounds was concentrated almost to dryness by distillation *in vacuo* and the residue extracted first with petroleum ether and finally with methanol.

The bicarbonate- and carbonate-extractable fractions were acidified and extracted with ether, then with trichlorethylene, after which the aqueous residues were re-extracted into cyclohexanone. The solvents were distilled off under reduced pressure. All the fractions were therefore water-soluble, non-volatile components of the glucose-glycine reaction mixture. Results from a typical extraction of a 250-ml quantity of the glucose-glycine mixture, during incubation at 50°C, are given in Table 1.

Table 1. Water-soluble non-volatile extracts from glucose-glycine solution incubated at 50°C.

Extract	Weight (mg) of extracted solids after incubation period of:		
	3 weeks	4 weeks	5 weeks
Acid fractions			
Ether-soluble	60	148	71
Trichlorethylene-soluble	trace	10	105
Cyclohexanone-soluble			
1) L.P.-soluble	100	268	260
2) MeOH-soluble		147	140
Phenols and weak acids			
Ether-soluble	7	41	40
Trichlorethylene-soluble	Nil	1	15
Cyclohexanone-soluble			
1) L.P.-soluble	5	47	90
2) MeOH-soluble		31	40
Neutral fractions			
Ether-soluble	31		
Trichlorethylene-soluble	5	87	101
Cyclohexanone-soluble			
1) L.P.-soluble	20	295	493
2) MeOH-soluble		136	174
Total	228 mg	1211 mg	1529 mg

L.P., light petroleum, b.p. 60–80°C.

An aliquot from each organic solvent fraction was blown free of solvent in a stream of air and the product dissolved in water. These solutions were then treated with an acid solution of 2,4-dinitrophenylhydrazine hydrochloride. The cyclohexanone-soluble weak acid fraction did not give a precipitate, but all the other fractions did. All the cyclohexanone "neutral" fractions gave heavy precipitates.

No further fractionation was undertaken at this stage, but it was clear that the browning process involved a number of non-volatile water-soluble carbonyl compounds of differing properties.

Chromatography of 2,4-dinitrophenylhydrazones. Several fractions were examined, and preliminary column separations revealed a number of carbonylic constituents. The cyclohexanone-soluble neutral fraction was examined by column chromatography on Florisil, using carbon tetrachloride-chloroform and chloroform-methanol mixtures for development. At least twelve individual 2,4-dinitrophenyl-hydrazones were obtained from this one main fraction, some crystalline but not necessarily pure. About twenty of the subsidiary fractions were examined spectroscopically. Three gave peaks of 363–366 mμ, the majority between 367 and 398 mμ, whereas two or possibly three distinct, more complicated compounds existed in five fractions recording 402–411 mμ. Thus some of the constituents of the browning mixture appeared to be α,β-unsaturated carbonyl compounds.

Browning potentialities of divided solvent-extracted fractions. Samples of each fraction (2.5 mg) were blown free of solvent in a stream of dry air. The resultant solids were incubated: a) in a 2.5*M* solution of glucose (3.5 ml), and b) in a 1.0*M* solution of glycine (3.5 ml). The samples were incubated at 50°C and examined visually over a period of 2 months. All the fractions showed much greater rate of change, on visual examination, for the development of the yellow and brown colors when incubated with glycine than when incubated with glucose. Thus the presence of a nitrogen function provided a more rapid visible chromophore production.

Estimation of color development. Color development was followed visually. We have found this method to be at least as informative for the purpose required as reading on a colorimeter, as have Hodge and Rist (1953). In similar series we have followed the color development by both methods. Results are given in Tables 2 and 3, where the change in appearance is described as passing progressively through the stages straw → gold → orange → red brown → dark brown,

Table 2. Color development in glucose-glycine series.[a]

Sample no.	Additive	Appearance after incubation time: 2 days	6 days	12 days	20 days	34 days	62 days
B 1	Nil	Straw ++	Gold ++	Orange ++	R. Brown ++	D. Br. ++	D. Br.
B 2	Phosphate	Straw +	Gold +	Orange ++	R. Brown ++	D. Br.	D. Br.
B 3	Iron + phosphate	Straw 4+	Gold 4+	Orange 4+	R. Brown 4+	D. Br.	D. Br.
B 4	Iron	Straw 3+	Gold 3+	Orange 3+	R. Brown 3+	D. Br.	D. Br.
B 5	Sulfite	....	Straw ++	Gold ++	Orange ++	D. R. Br. +	D. Br.
B 6	Phosphate + sulfite	....	Straw +	Gold 4+	Orange +	D. R. Br. 3+	D. Br.
B 7	Phosphate + sulfite + iron	....	Straw 4+	Gold 4+	Orange 4+	D. R. Br. 4+	D. Br.
B 8	Iron + sulfite	....	Straw 3+	Gold 3+	Orange 3+	D. R. Br. ++	D. Br.

[a] There was no color development in a similar series in which the additives were incubated in glucose without the addition of glycine. Gold, full, deep yellow; Orange (Or.), deep orange; R, red; Br., brown; D, deep; deep brown, opaque brown. "Plus" indicates intensity.

Table 3. Color development in sucrose-glycine series.

Sample no.	Additive	Appearance after incubation time: 2 days	6 days	12 days	20 days	34 days	62 days
B 12	Nil	....		Straw ++	Gold 4++	D. R. Br. 4+	D. Br.
B 14	Phosphate	....		....	Straw ++	D. R. Br. 4+	D. Br.
B 15	Sulfite	....		....	....	Gold +	R. Br. +
B 16	Phosphate + sulfite	....		....	....	Gold 3+	R. Br. 3+

Gold, full, deep yellow; Orange (Or.), deep orange; R, red; Br., brown; D, deep; Deep brown, opaque brown. "Plus" indicates intensity.

[There was a slight color development after 12 days in a sucrose-phosphate mixture similar to B14, containing no glycine. This may have been due to the accelerating influence of phosphate and the early browning potential of fructose (Burton and McWeeny, 1963a).]

the solution becoming opaque to transmitted light in the final stage.

RESULTS AND DISCUSSION

Haas *et al.* (1948) had shown that their ethyl acetate extract of fruit product browning mixtures had peaks around 285 mμ, in the region in which furfurals absorb, and Anet (1959) showed peaks in a similar region for the decomposition of difructose-glycine and the carbonyl decomposition products of difructose-glycine. In Anet's experiments the levels obtained were much lower in the presence of sodium metabisulfite. The absorption at 285 mμ was thought to be due to furfural compounds, though Stadtman (1948) mentioned the presence of 13 other unknown carbonyl components in his browning fruit products.

In the experiments reported here there was a marked initial lowering of the level of chromophore development, fluorescence and of the level of conjugated unsaturated carbonyl compounds in the early stages of the reaction when sulfite was present.

When phosphate was added alone, or with sulfite, there was an initial slight depression during the first 14 days, in the browning rate when the pH was allowed to drop, but this effect has not been noticed in subsequent experiments where the pH was kept constant, so that the lower browning rate was probably due to a pH effect. We are now aware that the presence of phosphate generally increases the rate of early sugar-derived chromophore development in model systems and in stored vegetable pulps, and that a decrease in pH slows the rate (Burton *et al.*, 1962b). There did appear to be an enhancement of the rate of appearance of conjugated unsaturated carbonyl compounds during this period (see graphs), irrespective of the lag in browning caused by the pH drop. After this short initial phase, phosphate greatly increased the browning rate and the rate of development of the conjugated unsaturated carbonyl and fluorescent compounds. This effect became marked after about 30 days' incubation. [Calcium has an opposite effect in that it tends to slow up sugar-amino chromophore development but not phosphatide-amino browning (Burton and McWeeny, 1963b) or carbonyl amino browning.]

Iron caused increased browning at the level used, with an increase in the quantity of conjugated unsaturated carbonyl compounds and fluorescence. The comparative slight initial superiority of phosphate as a browning agent over iron, at the concentrations used, was abolished later on in the course of the reaction, which suggests that phosphate exerts an initial effect which cannot be induced by iron, and that an accelerating effect due to the iron occurs later. The phosphate activity observed may well be due to its effect on the sugar, as mentioned by Burton and McWeeny (1963a), whereas the effects found with iron may be partly due to chelation and catalytic development of carbonyl compounds and red pigments. We have found that some metal ions may be incorporated into the polymeric material in analogous model systems, and it is worth noting here that we have found that a gray color may develop in stored $FeSO_4$-treated dehydrated vegetables (potatoes).

Little furfural appeared in these experiments until quite late, when the conjugated unsaturated carbonyl compounds were present in quantity. This suggests that furfurals are produced only in relatively small quantities in model mixtures containing a fair proportion of a nitrogen function, and that browning probably occurs by reaction of more reactive unsaturated carbonyl compounds.

An explanation of the phenomena occurring in the glucose-glycine and sulfited glucose-glycine systems has been given by Burton *et al.* (1962a,b,c,d) and by Burton and McWeeny (1962), in which it was postulated that early unsaturation occurs, and that chromophores can develop as a result of reaction between water-soluble unsaturated carbonyl compounds and nitrogenous compounds. In this work we examined the Maillard reaction occurring in an incubated glucose-glycine system, using a multiple series of spaced additions of ^{35}S-sulfite with colorimetric, spectrographic carbonyl, Monier-Williams SO_2, and sulfate determinations, and ^{35}S chromatogram scan-

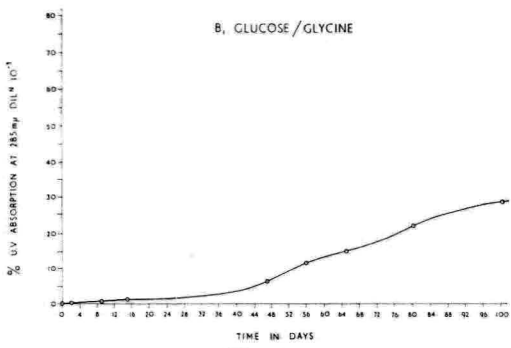


Fig. 1.

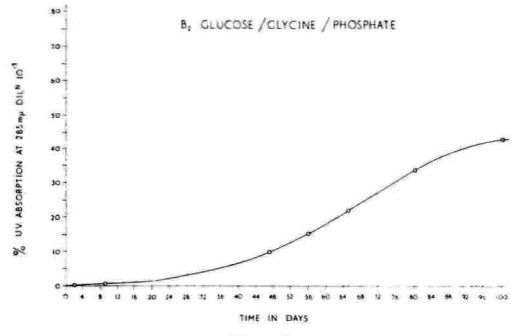


Fig. 2.

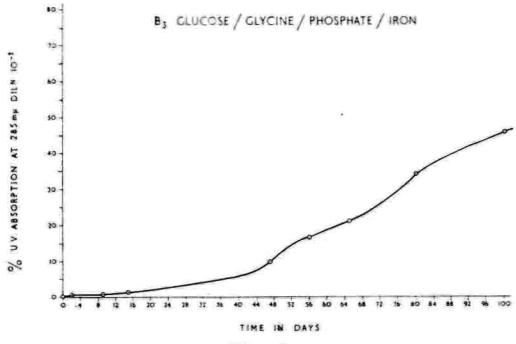
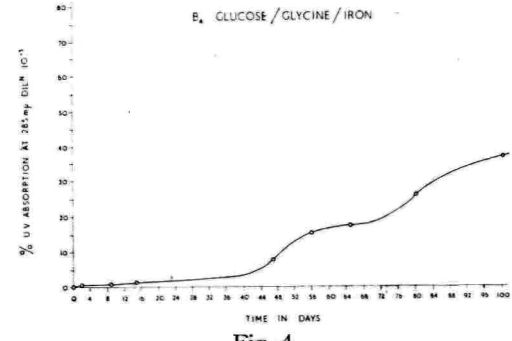


Fig. 3.



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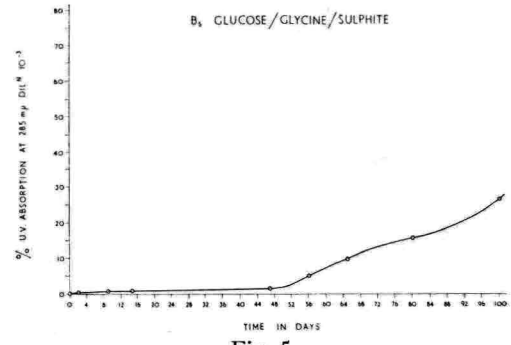

Fig. 5.

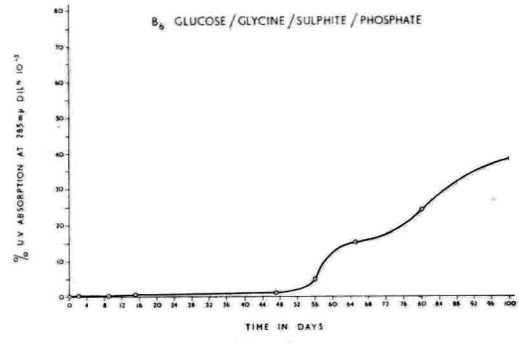

Fig. 6.

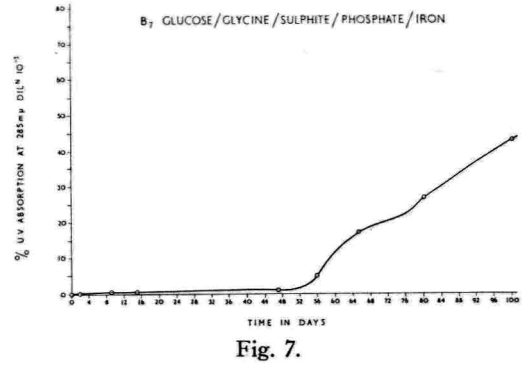

Fig. 7.

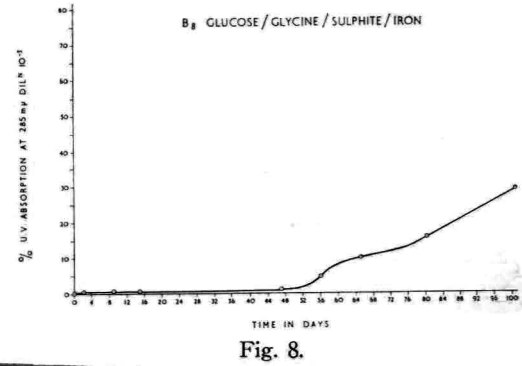

Fig. 8.

ning in order to determine the fate of the sulfite. This work showed that the rate of early chromophore development was more dependent on the amino acid concentration than on that of the sugar.

Haas *et al.* (1958) stated that continuous extraction of the browning fruit mixture with ethyl acetate retarded the browning, and Wahhab (1948) and Stadtman (1948) showed that the ethyl acetate contained an overwhelming proportion of furfural.

Yoshihiro *et al.* (1961) reported that furfurals cause little increase in browning when added to model systems, with a slow utilization of alpha-NH_2 groups, and this aspect is dealt with in more detail in a current paper (McWeeny and Burton, 1963). Kato (1960) also found that HMF was not important in his sugar-amino browning reactions.

We have here examined the glucose-glycine reaction in a more complex manner, using spaced extractions with ether, trichlorethylene, and cyclohexanone to determine the quantities of extractable material in solution at successive stages of the reaction, in order to examine, among other things, the development of the larger, darker compounds from the smaller, less colored

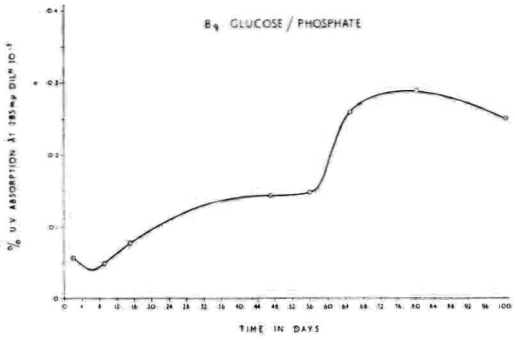

Fig. 9.

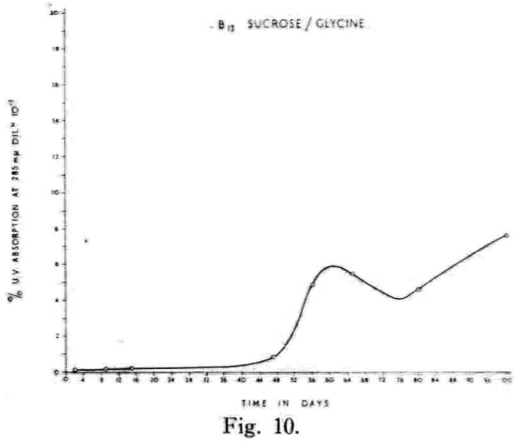

Fig. 10.

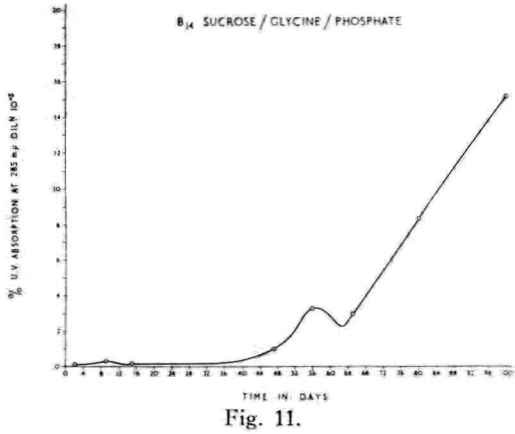

Fig. 11.

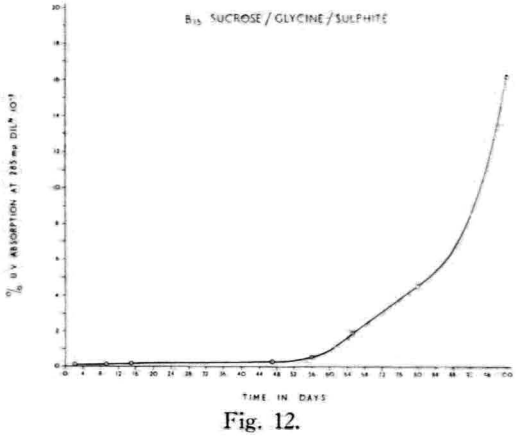

Fig. 12.

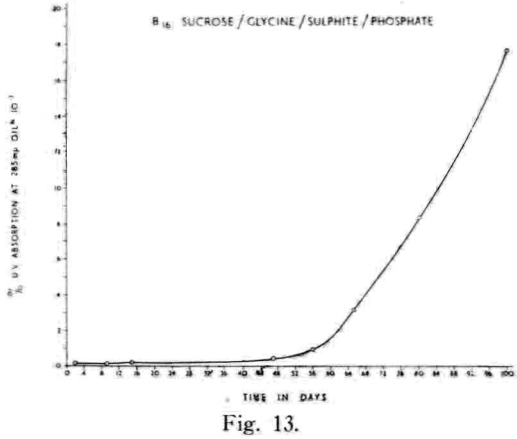

Fig. 13.

compounds. The extracts were each divided into acid, weak acid, and neutral compounds, as shown in Table 1.

From these results it appears that the quantity of water-soluble non-volatile compounds extractable with these solvents increases with time, and the increase is most rapid at a point where the unsaturated carbonyl content, color, and fluorescence is also developing at a fast rate (*cf.* Burton *et al.*, 1962a). It is also clear from the quantity of the cyclohexanone-extractable material that is soluble in light petroleum, that much dehydration had occurred to produce compounds with these physical characteristics. Much of the cyclohexanone-extractable fraction is revealed as neutral in nature. As incubation proceeded, the viscosity of the reaction mixture increased, and by the fifth week some of the product had gelled, indicating that some otherwise-extractable material had become incorporated into cross-linked polymeric material.

Most of the fractions are fluorescent and contain carbonyl compounds, and a spectroscopic examination of well separated fractions of the 2,4-dinitrophenylhydrazone mixture revealed that a multiplicity of water-soluble unsaturated carbonyl compounds are present in the system. While this phase of the work was in progress, Ellis *et al.* (1961), Buttery *et al.* (1961), and Yu *et al.* (1961) reported that they had found a large number of carbonyl compounds from potato granules, bread, and fish oils. A number of these carbonyl compounds were shown to be unsaturated, and ene-dials and 2,4-dieneal structures were postulated. Some of our darker extracts give brownish precipitates on reaction with 2,4-dinitrophenylhydrazine. All the carbonyl fractions isolated by us cause a more rapid development of color when incubated in fresh glucose-glycine mixtures, and all show a more rapid color development when incubated with glycine as opposed to glucose. This shows clearly that at the relative concentrations of glucose and glycine used, the chromophore development is more sensitive to glycine than to glucose concentrations.

It appears that although in the early stages of glucose-glycine incubation a number of small carbonyl compounds are present, and that these can be extracted into ethyl acetate, as Haas *et al.* (1948) have done, the subsequent course is for these compounds to develop into larger, more light-absorbent molecules, many of which are themselves still fluorescent and still carbonyl compounds (*cf.* Burton *et al.* 1962d). The course of the reaction described here is indicated by comparing the rate of development of conjugated unsaturated carbonyl and the quantities, colors, and physical properties of the products at each time interval. The fact that the cyclohexanone-extractable materials (which are not found very early in the reaction and which increase in quantity with time) are largely soluble in light petroleum would seem to show that they are in fact small polymeric highly dehydrated compounds. During the course of writing we have noted the results of Pollock and Siefker (1956) and Siefker and Pollock (1956). From inspection of the curves they published, it appears that in their heated sugar-glycine systems both the reaction solution and the more polymeric undialyzable fractions obtained as the reaction progressed were multi-carbonylic in nature, with solution viscosity and percentage of undialyzable matter increasing with time. This indicates that the intermediates (which varied with the sugar) were still building up a carbonyl-type polymer. In the aldose-glycine system, therefore, we know that browning occurs following a drop in α-NH_2 content, with a development of and increase in the level of conjugated unsaturated carbonyl compounds (which themselves brown more quickly in the presence of glycine), and with concomitant reduction in free α-amino nitrogen and increase in fluorescence. There is also a decrease in pH, an increase in percentage undialyzable material and solvent-extractable compounds, and in viscosity. We found that the viscosity of comparable aldose-amino solutions was less in the presence of sulfites than in their absence. A similar state of affairs (in some respects) appears to exist in the development of chromophores in milk products (Tarassuk and Simonson, 1950; Simonson and Tarassuk, 1952).

The number of reactive compounds produced even in a simple glucose-glycine system is quite large, and these reactive com-

pounds appear to link up to form larger molecules. The mechanism of browning therefore appears to be more complicated than the monofructose-glycine → difructose-glycine → 3,4-dideoxy 3,4-unsaturated hexosone → HMF system of Anet and his collaborators (1957–62; *cf.* McWeeny and Burton, 1963).

In the experiments recorded here, the analogous reaction with sucrose followed an apparently similar path, with the difference that in the early stages of browning there were indications of a rapid utilization of the fructose formed. This would not be incongruous with our later findings in the field of ketose amino reactions (*cf.* Burton and McWeeny, 1963a; Burton *et al.*, 1963).

ACKNOWLEDGMENTS

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The Organic Constituents of Food. II. Celery[a]

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INTRODUCTION

Celery (*Apium graveolens*, L.) (Umbelliferae) ranks with the more important vegetables used in the United States today; the annual per capita consumption is about eight pounds. Although several states produce commercial quantities, the California crop, worth $29,413,000 in 1960, represented over 57 percent of the U. S. Supply.

The stalks (or stems) of the plant are the part normally eaten, usually in the fresh state. Cooked, celery has found some favor in such food preparations as soups and stews. Celery seed, however, and the seed oil have been used for many years as flavoring materials. At present there is a trend toward increased processing of celery to utilize it in juice blends, soups, and other processed vegetable products because of its unique flavor. This trend has recently accelerated because of the uncertain market for the fresh produce. Gold and Wilson (1961) report that in 1959, 82,500,000 pounds of celery (some 48% of the Arizona crop and 15% of the Florida crop) was not marketed, because of lack of demand. The use of celery in food blends for its unusual flavor has greatly increased the interest in the nature of its chemical constituents.

Celery is a close relative of parsnip, carrot, and parsley. A wild form still grows as a low herb in the marshes of the coastal regions of the Mediterranean and bears little resemblance to the plant cultivated commercially. The wild plant is bitter—even poisonous—and was probably used for medicinal purposes before it was used as a food. With respect to its curative value, Phillips (1827) stated:

> "Both the roots and seeds are used medicinally, especially in obstructions of the liver and spleen; they warm and dry; they purify, attenuate, and carry off fevers, jaundice, and diarrhea . . . a conserve of blanched celery is good for pains in the chest and windy colic and a decoction or tea is a diuretic that is said to relieve the gravel."

At present, however, no important medical value is ascribed to celery.

From the wild celery, horticulturists have developed the two familiar garden varieties —"dulce," the common celery, and "rapaceum," the root vegetable celeriac. The Romans used as food a celery that was probably not very different from the wild form. They considered it another form of parsley and named both plants *apium*, from *apis*, the bee, because of the partiality bees had for the blossoms.

To avoid the confusion that surrounds some of the early chemical work on celery, a few further words about its history are in order. The French modified the name to *ache* (no relation to pain); medieval England already accepted this word for parsley, but Phillips (1827) described it as the true English name for celery. However, to distinguish between celery and parsley, celery was called "small ache." This soon became contracted to "smallage," a name that was quite acceptable through the 19th century. The present name was originally spelled sellery by the English, and is derived from the Greek work for parsley, *selinon*.

Actually, celery was not commonly used in England in medieval times, and the first mention we have of its cultivation and use as a food plant was in France, in 1623. At present, its habitat extends from Sweden southward to Northern Africa and into the mountains of India, as well as over much of North America. Both green and self-blanching types are grown, but the literature does not generally specify which form was under investigation. Several parts of the plant

[a] The first article in the series (Crosby, 1963) describes the purpose, scope, and organization of the series.

have been investigated for their chemical constituents, and this anatomical information, when available, is presented herein.

COMPOSITION

Macromolecular components. The macromolecular components of celery account for the greatest part of the dry weight. They are generally described in terms of a so-called "proximate analysis," typical values for which are given in Table 1. In such

Table 1. Proximate composition of celery (Howard *et al.*, 1962).

Variety	Water	Protein	Fat	Total sugar	Other Carbohydrates
	Percent of fresh weight				
Self-blanching	96	0.7	0.1	1.0	0.2
Green	95	0.9	0.1	1.0	0.2

analyses, the term *water* actually represents not only water but all other easily volatilized substances including the lower alcohols, amines, aldehydes, ketones, hydrocarbons, and many of the fatty acids. Until recent years, the chemistry of this volatile fraction has usually been ignored.

Protein is usually determined by multiplication of percentage of nitrogen found in the sample by a factor (generally 6.25) that has been shown to vary considerably among plant species. Consequently, all nitrogen-containing constituents, such as nucleic acids, alkaloids, and porphyrins, are included in addition to true protein. However, since protein generally constitutes a large proportion of the nitrogenous solid matter, the error so introduced is probably comparatively small in most cases. Although celery proteins represent about 16 percent of the dry weight of a typical sample, no further characterization of these substances appears to have been made. In fact, not even the component amino acids have been investigated in detail, although it is to be presumed that the normal array would be present. The compilation of Orr and Watt (1957) gives analytical data for only 5 amino acids—tryptophan, lysine, methionine, cysteine, and tyrosine, and these from only one sample. This lack of information about celery seems to prevail for many other vegetables as well, and is rather surprising when one considers that the amino acid content has been determined for many much less common plants.

Carbohydrate content is usually calculated as the percentage necessary to bring the sum of the component protein, ash, and fat to 100 percent. Obviously, figures derived in this way are open to serious error, and direct measurement of the various forms of true carbohydrate becomes of considerable importance. The figures in Table 1 were obtained by actual analysis.

Elwell and Dehn (1939) provided data on the pectic substances in celery, principally from the alcohol precipitation method. They reported that water-soluble pectin (5.72% of dry weight), easily hydrolyzable protopectin (6.28% of dry weight), and difficultly hydrolyzable protopectin (2.14% of dry weight) were present. The pectic acids in celery were not determined.

The work of Myers and Croll (1921) provides the only other available information on the carbohydrates in celery; values for "reducing" and "non-reducing" sugars were reported (Table 2). Hall (1957) also found reducing sugar to be present.

Table 2. Free sugars in celery expressed in terms of glucose (Myers and Croll, 1921).

Type of sugar	% of fresh weight	
	Outside parts	Hearts
Reducing sugar	0.54	1.70
Non-reducing sugar	0.02	0.24
Total	0.56	1.94
Total carbohydrate (including fiber)	3.3	3.3

No information has been published on other macromolecular components such as nucleic acids, lignins, or tannins.

Common components of low molecular weight. Of the simple sugars that one would expect to occur in celery, only sucrose and glucose have actually been identified (Myers and Croll, 1921). Despite the fact that no other sugars have been isolated, there is little question that the common pentoses, hexoses, disaccharides, and other simple sugars are present. There is one report of mannitol having been detected in the roots (Anon., 1923).

An unusual sugar, apiose (I), has been obtained from celery, but it probably exists in the plant, in combination with flavones, in the form of glycosides (Farooq *et al.*, 1958). Chemically, apiose is 3-*C*-(hydroxymethyl)-D-*glycero*-aldotetrose. Gorin and Perlin (1958) and Raphael and Roxburgh (1955) have shown by synthesis that the naturally occurring form of this sugar is the D-isomer and has an open-chain structure. The chemistry of apiose has been reviewed by Hudson (1949) and Shafizadeh (1956). More will be said about the apiosyl glycosides in a later section.

Only one reference to the free amino acids of celery was found in the literature. Rockland (1959) provided evidence by paper chromatography that the following non-protein amino acids were present: alanine, glutamic acid, aspartic acid, serine, γ-aminobutyric acid, glutamine, and asparagine. Tyrosine was reported to occur in the celery root (Bamberger and Landsiedl, 1904), but there is doubt about whether it was present in the free state or formed by hydrolysis of protein during isolation.

The term *fat* in the proximate analysis (Table 1) generally includes all ether-extractable substances, but significant quantities of true fats and related substances undoubtedly constitute a major proportion of this fraction. Christian and Hilditch (1929) described the physical properties of celery seed fat and estimated its composition by saponification, although the original glycerides themselves were not identified.

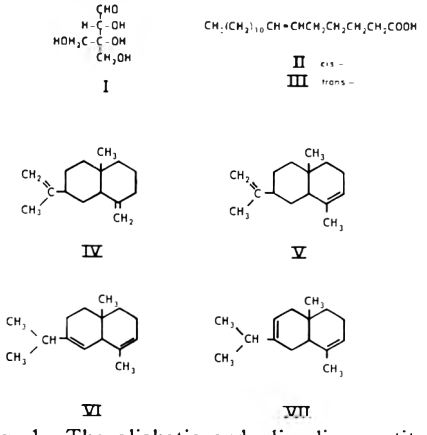

Fig. 1. The aliphatic and alicyclic constituents of celery.

Several investigations (Farooq *et al.*, 1953; Kurono and Sakai, 1959; Gold and Wilson, 1961) have shown specific fatty acids to be present in the seed oil; as might be expected, oleic, linoleic, and palmitic acids were isolated in addition to the less common petroselenic acid (II) and petroselaidic acid (III). By gas chromatography and infrared spectral techniques, several shorter-chain fatty acids also were identified: isobutyric acid, valeric acid, and an unidentified branched-chain heptanoic acid. From the low acidities reported for celery fat, it appears probable that these acids exist to a major extent as glycerides in the plant rather than in the free form *per se*.

It seems inevitable that other acids such as those involved in the tricarboxylic acid cycle should be present. However, it was not until 1961 that Schramm (1961) detected, by paper chromatography, citric, isocitric, succinic, fumaric, malic, and tartaric acids in celery roots. Maleic and citric acids had been shown earlier to occur in the edible portion (Hartmann and Hillig, 1934), and pyruvic acid was found by Gold and Wilson (1961). Bentley (1952) showed malonic acid to occur in the leaves, and glycolic acid was reported by several investigators, including Andrews and Viser (1951), who determined that it was present in the amount of 0.034%.

As reported in Table 3, celery does not appear to be a particularly good source of any of the vitamins, though a number of the B vitamins are present. No report has been made of the presence of either vitamins A, K, or D. Harris *et al.* (1950) reported that fresh celery contains 0.48 mg/100 g of mixed tocopherols, of which more than 96% was α-tocopherol.

Table 3. The vitamins of celery (Burton, 1959; Sebrell and Harris, 1954).

Vitamin	μg/100 g	Vitamin	μg/100 g
E	480	B_6	154
B_1	500	Folic acid	7.2
B_2	400	C	7000
Niacin	400	A	0

Unusual or specific components. *Aliphatic and alicyclic compounds.* Very few simple aliphatic compounds have been iso-

lated from celery. *n*-Heptanol and *n*-octanol were obtained from an extract of the entire aboveground portion of the plant by Gold and Wilson (1961) and characterized by gas chromatography and infrared spectra. (*n*-Octanol is generally considered to possess a disagreeable odor, even in moderate concentrations.) The common terpene, limonene, was shown by the same workers to be present to the extent of 80–90% in the oil fraction, as were the open-chain isomer, myrcene, and the related terpene aldehyde, citral (neral). The terpenoid pigment "carotene" has been reported (Wilcox and Galloway, 1951; Wolf, 1955), but no further identification as to type was given. Strangely, no report could be found of the occurrence of the common hydrocarbons phytoene and phytofluene.

Ciamician and Silber observed in 1897 that celery seed oil contained a sesquiterpene, $C_{15}H_{24}$. This compound was further characterized by research at Schimmel and Co. (1910) through the preparation of a crystalline dihydrochloride, and was given the name "selinene." Semmler and Risse (1913), in confirming the work at Schimmel, determined that the naturally occurring selinene differed in physical properties from the "selinene" obtained by regeneration of the dihydrochloride derivative. They designated the naturally occurring one as β-selinene and the other as α-selinene, and assigned the formulas (IV) and (V), respectively. Such great names as Ruzicka, Semmler, Stoll, and Linstead have been associated with the efforts to elucidate the structures of the selinenes. Simonsen and Barton (1952) discussed the various chemical reactions (oxidation, reduction, ozonolysis, etc.) involved in determining their structures. Accumulated evidence indicates that formulas IV and V are finally correct. It appears that α-selinene arises by the generation of an endocyclic double bond upon elimination of hydrogen chloride from "β-selinene dihydrochloride."

Three other selinenes have been reported. Simonsen and Barton (1952) offer formulas for δ-selinene (VI) and ϵ-selinene (VII), but a search of the literature failed to reveal the chemical structure of the γ-selinene proposed by Ognyanov and Ivanov (1958).

Workers at Schimmel and Co., in 1910, also mentioned that sesquiterpene alcohols ($C_{14}H_{26}O$) were present in celery seed oil. Ruzicka and Stoll (1923) later observed the same type of alcohols in the oil, but no identification was made; the literature is silent on any subsequent work on the subject. Only one reference to the steroids (Wall and Kelley, 1947) could be found. These workers attempted to determine the nature of the leaf sterols of several plants by the production of color in the Libermann-Burchard reaction. They demonstrated the presence of a phytosterol (144 mg per 100 g of dehydrated meal prepared from celery tops) that could not be classed in either the sitosterol or spinosterol group. No attempt was made to elucidate its chemical nature further.

Aromatic compounds. Of the simple phenolic substances, only guaiacol has been shown to occur in celery (Gold and Wilson, 1961), although a trace of unidentified "phenols" was reported (Makarova and Borisyuk, 1957) in the residue remaining after distillation of celery oil. However, two interesting phenolic ethers known as apiole [1-allyl-2,5-dimethoxy-3,4-methylenedioxybenzene (VIII)] and myristicin [1-allyl-3-methoxy-4,5-methylenedioxybenzene (IX)] have also been isolated (Karmazin, 1955). Both substances had been reported to occur in parsley and other plants, and their structures had been determined satisfactorily many years earlier. These compounds obviously are closely related to the common flavoring agent safrole (1-allyl-3,4-methylenedioxybenzene), which is currently in disrepute because of alleged carcinogenicity.

It appears that the apiole that Karmazin detected in celery indeed has the structure VIII, but a note of caution should be interjected at this point. The apiol(e) originally obtained from plant sources undoubtedly was a mixture of several compounds. English and French "apiole" is said to consist mainly of myristicin, whereas German "apiole" primarily contains stearoptene (the portion of a natural essential oil that separates as a solid on cooling or long standing) (Small, 1948). The highly toxic nature of the "apiole" that was used medicinally for

many decades arises primarily from the presence of the glycoside apiin (Castagnou and Quilichini, 1952). To confuse the subject further, an isomer of apiole has been isolated from dill and other plant sources and named dillapiole (1-allyl-2,3-dimethoxy-4,5-methylenedioxybenzene). Still another isomer, isoapiole (1-propenyl-2,3-dimethoxy-4,5-methylenedioxybenzene), has been reported in other plants, but no reference has been made to it for some 20 years.

It seems certain from physical data that the true apiole described by present-day workers does have the formula VIII as shown, but a great deal of discretion and discernment must be exercised in reading earlier literature describing "apiole."

Very few other aromatic compounds have been reported to occur in celery. Paper chromatography, coupled with fluorescence detection, has indicated the presence of the common aromatic compounds *p*-coumaric acid, caffeic acid, ferulic acid, chlorogenic acid, and isochlorogenic acid (Herrmann, 1957). Several phthalide derivatives from celery will be discussed in the next section.

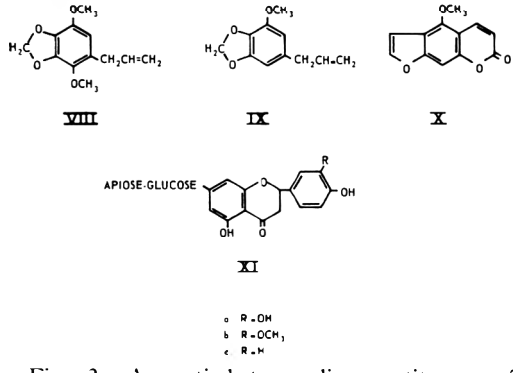

Fig. 2. Aromatic-heterocyclic constituents of celery.

Oxygen-containing heterocyclic compounds. Compounds containing oxygen in a ring system have long held considerable interest for the chemist and biochemist because a great many of them possess significant biological activity; coumarin derivatives are no exception. Herrmann (1957) suggested the presence in celery of scopoletin by chromatographic and fluorescence techniques, but no other simple coumarins have been reported.

It has been known for some time that celery could cause a dermatitis on the hands and arms of people who handle it in the fields, but the etiology was unknown. Based on knowledge of the biological effects in the presence of sunlight of other plant extractives known to contain coumarin derivatives, Musajo *et al.* (1954) were able to isolate from celery the known furocouramin 5-methoxypsoralen, also called bergapten (X). This work was further substantiated by Rodighiero and Allegri (1959). Bergapten and many other furocoumarins possess the property of sensitizing the skin to sunlight (ultraviolet light). This may explain the use of *Ammi majus* by the Arabians for treatment of lack of skin pigmentation and *Psoralea corylifolia* by people of India for the treatment of vitiligo—these plants have yielded several other furocoumarins as well as bergapten (Musajo *et al.*, 1954).

The glycosides represent a class of substances found in most plants—compounds formed by the combination of one or more molecules of a simple sugar with another moiety referred to as an aglycone. The aglycone portion of the molecule generally contains a free hydroxyl group that is attached to the sugar in a hemiacetal type linkage. Flavones frequently serve as the aglycone. Because the glycoside linkage is rather labile, hydrolysis may occur during extraction of the plant materials, resulting in artifacts.

Several flavone glycosides from celery have been studied; all of those reported have the same sugar arrangement and differ only in the aglycone portion. The carbohydrate portion consists of the five-carbon sugar apiose, mentioned previously, in combination with glucose. In 1953, Farooq *et al.* isolated two glycosidic fractions from celery seed and named them graveobioside A and graveobioside B. By stepwise hydrolysis, first apiose and then glucose were obtained from both glycosides. This served to prove that glucose was attached directly to the aglycone. Likewise, those workers were able to show that complete methylation of either graveobioside A or B followed by hydrolysis yielded the 3′,4′,5-trimethyl ether of luteolin. This established the point of attachment of the sugar at the 7-position of

the aglycone. Thus they were able to formulate graveobioside A as the 7-apiosylglucoside of luteolin (3′,4′,5,7-tetrahydroxyflavone) and graveobioside B as the 7-apiosylglucoside of chrysoeriol (4′,5,7-trihydroxy-3′-methoxyflavone), respectively represented by formulas XIa and XIb.

At about the same time that celery was being investigated by Farooq *et al.*, the glycoside apiin from parsley was being studied by Hemming and Ollis (1953) and by Nordström *et al.* (1953). Both of these groups, using hydrolytic and methylation procedures analogous to those mentioned above, were able to show that apiin was the 7-apiosylglucoside of the flavone apigenin (4′,5,7-trihydroxyflavone) (XIc). This discovery led to reinvestigation of the nature of graveobiosides A and B. With paper chromatographic techniques, graveobioside B was resolved into two components, which were identified as apiin and chrysoeriol-7-apiosylglucoside (XIb) (Farooq *et al.*, 1958).

The attachment of the sugars at the 7-position is only partly known. It seems certain that the linkage of the flavone with glucose is at the 1-position of the sugar, and there is evidence that it has the β-configuration (Hemming and Ollis, 1953) to result in flavone-7-apiosyl-β-glucosides. Although evidence was presented (Gorin and Perlin, 1958) that naturally occurring apiose exists in the open-chain form, Hudson (1949), in discussing the nature of apiose and apiose glycosides, suggests that the apiose exists in glycosides in the furanose form. However, he concedes that the specific structure of the apiofuranosyl radical and its point of attachment to the glucose moiety must wait for later determination.

Two rather unusual compounds were isolated from celery seed oil by Ciamician and Silber (1897). These substances were given the names of sedanonic anhydride and sedanolide. By a series of reactions described in the original literature, formulas XII and XIII were respectively assigned to them; the chemistry of the two lactones and the corresponding acids, sedanonic acid (XIV) and sedanolic acid (XV), was examined in detail. Similar compounds have been isolated from other genera of Umbelliferae (Karrer, 1958). The evidence presented for sedanonic acid and its lactone seems adequate to substantiate their formulas, although there is still some question about the position of the endocyclic double bond. Even less convincing is experimental evidence for the position of the double bond in sedanolic acid and sedanolide. Sedanonic acid appears to be quite stable (Ciamician and Silber, 1897), but Naves (1943), from further work on related compounds, reported that sedanolic acid is somewhat unstable and is readily converted into sedanolide.

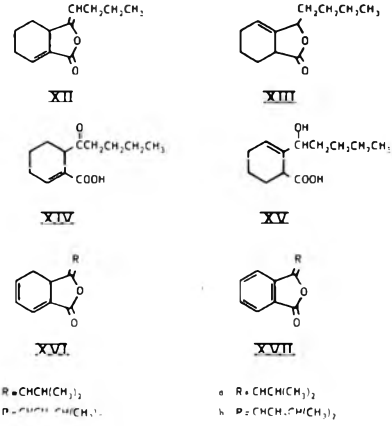

Fig. 3. The phthalide derivatives in celery.

Small (1948) claims that the characteristic odor and flavor of celery are due to a mixture of sedanonic anhydride and sedanolide. Further evidence of this was found by Gold and Wilson (1961) in their study of the odoriferous substances in celery. They indicated that sedanonic anhydride and sedanolide have a definite celery odor, but they were quick to point out that the aroma is caused by a blend of compounds in a particular proportion. In a more recent report, Gold and Wilson (1963) described the isolation, by gas chromatography, of four other phthalide derivatives. From retention times and infrared and ultraviolet spectral data, they were able to identify 3-isobutylidene-3a,4-dihydrophthalide (XVIa), 3-isovalidene-3a,4-dihydrophthalide (XVIb), and traces of the corresponding aromatic analogs (XVIIa and XVIIb). The structures of the last two compounds were authenticated by synthesis. With respect to aroma, those workers suggested a relation between odor

and chemical structure; they found that when one of the hydrogens on the γ-carbon atom of a $\Delta^{2,6}$- dihydro-, a Δ^{3}-tetrahydro-, or a hexahydrophthalide was replaced by an alkyl group, a celery odor was noted.

Makarova and Borisyuk (1957) claimed to have isolated both sadanonic and sedanolic acid in trace amounts from the distillation residue of the seed oil. It seems probable that the free acids, if present in celery, do not contribute in any major way to the typical celery odor.

Nitrogenous compounds. Apparently the presence of chlorophyll in green plants has been taken for granted because of their color and the acknowledged role of chlorophyll in photosynthesis. (Anyone who views the green vegetable in the grocer's display must concede that it contains chlorophyll). Despite the popularity of the chromatographic isolation of chlorophyll from green plants as a student demonstration, only one reference to its presence in celery could be located (Hall, 1957), and this did not indicate the type present. Only one other porphyrin, the metal-free relative of chlorophyll called pheophytin, has been reported (Willstätter and Oppé, 1911), and no other non-protein nitrogenous substances have been found in celery.

Miscellaneous compounds. An additional constituent has been reported, and it is placed in this category because of the lack of any information about its chemical nature. Marakova and Borisyuk (1957) mentioned the presence in seed oil of a substance that they designated as memonene, the chemical nature of which they did not describe. An extensive search of the literature failed to provide any additional reference to this "compound."

Another interesting substance, as yet to be identified, is the one responsible for bitterness in celery (Pan, 1961). It appears to be a non-flavonoid D-glucoside having a remarkably simple infrared spectrum and a rather low extinction coefficient in its ultraviolet absorption. Its structure has not yet been examined in detail (H. Pan, personal communication).

DISCUSSION

In reviewing our knowledge of the chemical constituents of celery, one is struck by the same curious circumstances that were noted in the review in this series on lettuce (Crosby, 1963). We really know very little about the naturally occurring substances to be found in this common food. What about nitrogenous compounds? Sulfur-containing compounds? Phosphorus compounds? For that matter, what about the common amino acids, vitamins, fatty acids, and the host of other simple, ordinary things that you might expect to have come to light in even elementary investigations of celery? Surely, a commodity worth some $50,000,000 annually to this country at the farm level deserves better treatment.

Obviously, by far the major part of the chemical research on this vegetable has centered on the seed oil, probably because of its past medicinal importance. A little is known about the constituents of the edible stalk, but almost nothing has been reported on the composition of the tops, although they are used occasionally as cattle feed. The closely related root product, celeriac, must be included in this same category—we appear to know virtually nothing about its constitution.

As with lettuce and other Compositae, the plant family (Umbelliferae) to which celery belongs has been the source of a wonderful variety of chemical substances. Most of the compounds isolated from members of the Umbelliferae are derived from the roots. Although celeriac continues to find use as food in this country, detail on root constituents of the family will be left to the forthcoming review of this series on the carrot. However, the aboveground portions of celery relatives can offer their share of interesting chemical structures, as shown in Table 4 and Fig. 4.

Several of the substances listed possess a high degree of physiological activity. Coniine and its related alkaloids, for example, are the toxic principles of the "poison hemlock" used throughout the ancient world for execution of prisoners and made famous to every school child for bringing about the death of Socrates. Khellin and related furochromones have long been used as antispasmodics, as vasodilators, and for a variety of other medicinal purposes (Huttrer and

Table 4. Selected constituents of the Umbelliferae.

Name	Plant species	Structure	Reference
Umbelliprenin	*Angelica archangelica* (seeds)	XVII	Späth and Vierhapper, 1938
Imperatorin	*Pastinaca sativa* (fruits)	XIX	Soine *et al.*, 1956
Coniine	*Conium maculatum* (leaves)	XX	Marion, 1950
Fenchone	*Foeniculum vulgare* (fruit)	XXI	Wallach and Hartmann, 1890
Khellin	*Ammi visnaga* (fruit)	XXII	Huttrer and Dale, 1951
8-Methylnonen-2-al-1	*Coriandrum sativum* (leaves)	XXIII	Carlblom, 1936
Creosol	*Pimpinella anisum* (seeds)	XIV	Monod and deDortan, 1950

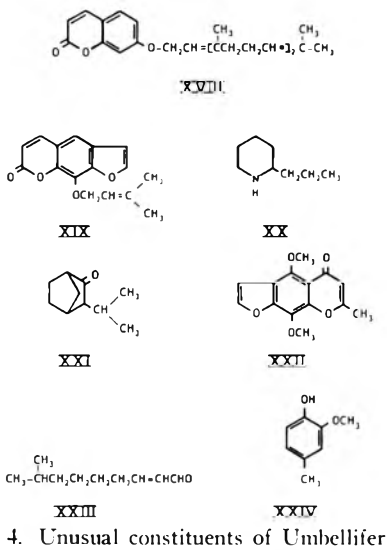

Fig. 4. Unusual constituents of Umbelliferae.

Dale, 1951). Imperatorin, like the bergapten of celery, has been shown to be an extremely potent fish poison. With this background, it seems inevitable that common celery must contain minor chemical constituents whose toxic properties have been neither recognized nor appreciated.

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Histological Analysis of Wound Healing in Potatoes Treated to Inhibit Sprouting
I. CIPC (Isopropyl-*N*-3-Chlorophenyl Carbamate) Treatments

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SUMMARY

Both wound periderm and sprouting were completely inhibited in freshly harvested cut potatoes treated by evaporation of CIPC (isopropyl-*N*-3-chlorophenyl carbamate) at 10 ppm and 100 ppm (based on tuber weight) and then incubated at 70–75°F and 88% relative humidity. At 1 ppm CIPC, sprouting at room temperature was only slightly retarded. Wound periderm was completely inhibited in the central tissues and markedly retarded in the cortex of the tubers.

Application of CIPC did not prevent suberization at the wound surface, but the walls of intact cells immediately subjacent were less suberized than those of the cut cells. Concomitant with inhibition of wound periderm, there was a marked enlargement of those cells that would normally have given rise to the wound periderm.

Variability of wound healing rates within different tissue zones of single tubers, and other variation between tubers, are considered significant to problems of sprout control and storage.

INTRODUCTION

The natural abilities of potatoes to sprout and to wound-heal have received much attention. Wound healing is a regenerative process and comprises both the suberization of cell walls and the formation of phellogen or cork cambium that produces new cells, wound periderm to the outside and an occasional phelloderm cell to the inside (Artschwager, 1927; Priestley and Woffenden, 1923). Sprouting, on the other hand, consists of the early growth and elongation of new stems from buds already preformed by embryonic tissue at the "eyes" of the tuber (Artschwager, 1924). This basic distinction results in different responses to sprout control treatments intended for extending storage and good keeping qualities.

Various problems and objectives are involved in sprout-control treatments of potatoes intended for processing. Increased sugar content in untreated tubers stored at low temperature (35–45°F) results in nonenzymatic browning in potato chips, frozen French fries, and dehydrated potato products. Storage of tubers at higher temperatures (50–60°F) appreciably reduces subsequent browning in their manufactured products, but sprouting and shrinkage losses are excessive. Treatments designed to inhibit sprouting are necessary, but they also inhibit wound periderm formation and may thus increase susceptibility to rot. Cunningham (1953) and Smith and Smart (1955, 1959) demonstrated that microorganisms can penetrate tuber tissues underlying wounds when the wound periderm tiers are not contiguous, even though suberization of the cell walls may be continuous over the wound surface.

Application of plant hormones, various other chemical agents, and irradiation treatments have been widely investigated as means of sprout control in potatoes (Perlasca, 1956; Sawyer, 1959). Several chemical treatments and irradiation have been found to inhibit sprouting effectively, but,

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because they also inhibit wound periderm initiation, it has been recommended that their application be delayed after harvest to ensure formation of a continuous wound periderm (Sawyer, 1959). Audia *et al.* (1962) have investigated the influence of isopropyl-*N*-3-chlorophenyl carbamate on wound healing and decay in treated Katahdin potato slices.

Most of the recently published information on wound healing seems to have been confined to results obtained from the cut surfaces of sliced tubers. The environmental conditions of such exposed surfaces differ from those of penetrating cuts or punctures, deep bruises and cracking, that are the types of wounds likely to be produced during harvest and handling conditions. Werner (1938) extensively investigated the effects of penetrating wounds upon storage of seed potatoes, described some of the histological aspects in detail, and thoroughly reviewed the earlier literature.

More recent advances in the development and use of fungicides and plant growth substances indicate a need for a reexamination of the histological aspects of healing in these natural wounds. A series of studies was undertaken at this laboratory during investigations on effects of inhibitor treatments on the processing qualities of potatoes. Preliminary results on studies of the influence of vapor-phase application of isopropyl-*N*-3-chlorophenyl carbamate (CIPC) on spoilage were reported by Hendel (1957). The present paper describes histological responses of cut halves of tubers treated with different concentrations of CIPC applied as a vapor. Effects of CIPC on healing of puncture wounds and on the growth of Fusarium inoculates will be described in a second report. A brief survey of salient histological features of wound healing in potatoes constitutes an additional objective of both papers.

MATERIALS AND METHODS

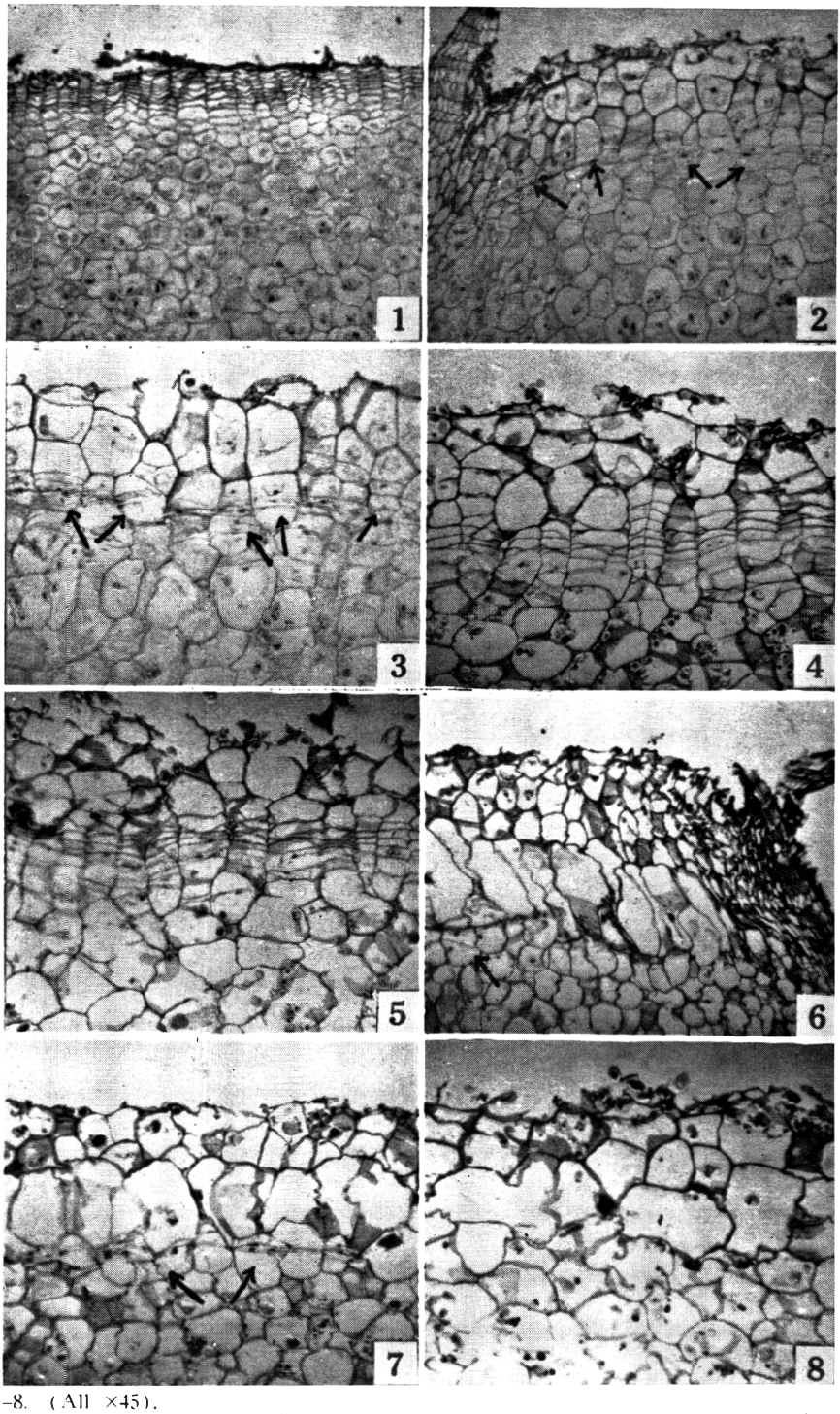
CIPC treatment. Freshly harvested California-grown Russet Burbank potatoes were carefully washed, then cut in halves transversely with an alcohol-flamed knife. Several halves of each were placed in 4-gallon cans and exposed to CIPC by evaporation (Sawyer and Dallyn, 1957) so that the samples received doses estimated at 1, 10, and 100 ppm (based on the weights of the tubers) after 24 hrs evaporation (Hendel, 1957). Controls received no CIPC. Each can contained a beaker of saturated barium chloride solution to hold relative humidity at approximately 88%. A hole of ½-in. diameter in each can lid provided some ventilation. The samples were then incubated at room temperature (70–75°F) in a closed room of about 50% relative humidity until histological sampling.

Histological preparation. The CIPC-treated samples were removed after 5, 10, and 18 days of incubation. Strips of tissue about 0.5 cm deep and 1 cm wide across the full tuber diameter were then removed from the cut surface of each half tuber. Each strip was further divided into 4 blocks, 2 representing the inner tissue zones of the tuber and 2 representing the outer, including the "vascular ring." These blocks were immediately killed and fixed in a solution of 10% technical formaldehyde and 8% acetic acid in 60% aqueous ethyl alcohol. After 24–48 hrs in the killing agent, they were dehydrated according to a standard alcohol-xylene schedule.

The samples were then infiltrated and embedded in soft paraffin (mp about 52°C). Blocks of embedded tissue were trimmed (to expose the tissue) and soaked in a softening agent consisting of 2 parts 60% ethyl alcohol and 1 part of glycerine and acidified with 3 or 4 drops concentrated HCl per 100 ml of mixture. Such soaking treatment appreciably reduced static and ribbon tearing of the starchy tissue during microtomy. Serial sections from each soaked block were cut on a rotary microtome at a thickness of 20 μ. The resulting ribbons of serial sections were divided into strips of 3–5 sections each. Approximately alternating strips were selected to make up 6–10 slides, representing most of the wound surface of each tissue block. From 4–8 tissue blocks were so sectioned from each half tuber.

The selected strips of sections were fixed to microslides with Haupt's gelatin adhesive, and, after removal of the paraffin, they were stained with Delafield's hematoxylin (10% ripened stock solution in tap water) and 0.2% safranin in 60% ethyl alcohol, differentiated in a conventional alcohol-xylene series, and mounted in a suitable resin. Good differentiation of suberized cell walls was obtained with the safranin.

Wound periderm was evaluated as to degree of continuity or percent of total linear distance in which the tiers of wound periderm were contiguous. The wound periderm of different tissue zones also was evaluated as to range in number of cells per tier for each environmental treatment and incubation period. These evaluations were



Figs. 1–8. (All ×45).

Fig. 1. Periderm or skin of a freshly harvested Russet Burbank tuber; note pronounced staining of suberized walls of outer cork cells and loosening of outermost, crushed cells.

Figs. 2–5. Wound periderm of control samples. Fig. 2. Wound periderm (arrows) near skin after 5 days' incubation; Fig. 3. Central zone of tuber after 5 days' incubation; Fig. 4. Cortical zone after 18 days' incubation; Fig. 5. Central zone after 18 days' incubation.

Fig. 6. Nearly complete inhibition of wound periderm near skin and cell enlargement with 1 ppm CIPC after 18 days' incubation (arrow lower left, indicates one initiating division).

Fig. 7. Wound periderm initials (arrows) and cell enlargement in cortical zone of tuber with 1 ppm CIPC treatment and 18 days' incubation.

Fig. 8. Central zone of tuber showing cell enlargement and complete inhibition of wound periderm with 10 ppm CIPC and 18 days' incubation.

based upon 2 or more series obtained from 10 or more sections each for each of the 4–8 tissue blocks, or over 100 cm of wound surface per sample. Care was taken to avoid a replication of results such as would likely occur with use of serial sections closely enough situated to provide cellular patterns of wound periderm that were either identical or nearly so. Because this type of evaluation provided clear-cut evidence for the histological intent of these investigations, no detailed statistical analyses were made.

RESULTS

Fig. 1 illustrates the outer periderm or skin of a freshly harvested, mature tuber. Six to 8 or 10 cells formed the tiers of the normal periderm in these Russet Burbank potatoes and the walls of outer, crushed cells were heavily suberized. Figs. 2–5, at the same magnification as Fig. 1, show the average degree of wound periderm formation in control samples after 5 and 18 days of incubation. Wound periderm was continuous in all controls after 5 days of incubation, but its development in terms of cells per tier was slightly more pronounced in the outer than in the inner tissue zones of the tuber (Figs. 2, 3). After 18 days, the wound periderm of the centrally located tissues had become nearly as well developed as that of the cortical tissues underlying the tuber skin (Figs. 4, 5).

Examinations of fresh sections cut by hand from control samples with 1–3 days of incubation showed that the initiating divisions occurred more rapidly in the cortical tissues than in the central tissues of the tuber. The continuity of newly formed tiers of 2–4 cells was usually established at least a day earlier in the cortical tissue than in the more central tissue zone. Rate of wound healing also varied between different tubers. Wound periderm was as well developed at 3 days of incubation in some tubers as in others at 5 days; and a few with 5 days of incubation had developed nearly as much wound periderm as most samples had developed between 10 and 15 days. However, all samples examined had tiers of wound periderm in which numbers of cells per tier for any given period of incubation, fell within the ranges listed in Table 1.

Exposure to 1 ppm CIPC resulted in nearly complete inhibition of wound periderm. Only occasional wound periderm initials were found, even after 18 days of incubation, and these were confined to the cortex of the tuber (Figs. 6, 7). However, a few half tuber samples incubated 18 days following treatment with 1 ppm CIPC showed nearly continuous wound periderm initials in the cortex and scattered initials in the inner tissues. This possibly could represent a partial recovery from an initial blocking of wound periderm formation—but it is also likely that the small amount of CIPC evaporated did not distribute uniformly over the cut surface of this half tuber. No wound periderm developed in any samples receiving 10 and 100 ppm CIPC (Fig. 8).

A striking characteristic of the samples treated with inhibiting levels of CIPC was enlargement of intact parenchyma cells close to the cut surface and apparently involving those cells that normally would undergo the initiating divisions for wound periderm (Figs. 6–8). These cells enlarged appreciably, and often elongated perpendicularly to the cut surface. An increase in average cell volume concomitant with the blocking of cell divisions as a result of X-irradiation at 5,000 R has been reported for cultures of animal cells actively dividing prior to treatment (Whitmore *et al.*, 1958). Except for Hendel's (1957) preliminary report on the present studies and the report of Audia

Table 1. Wound periderm development as influenced by CIPC (isopropyl-*N*-3-chlorophenyl carbamate) treatments.

CIPC (ppm)	Tissue region	Wound periderm development					
		5-day incubation		10-day incubation		15-day incubation	
		Percent continuity [a]	Range of cells per tier	Percent continuity [a]	Range of cells per tier	Percent continuity [a]	Range of cells per tier
0	near skin	100	2–6	100	4–8	100	4–10
	cortex-vascular ring	100	4–6	100	4–10	100	6–14
	central	100	2–6	100	2–6	100	4–10
1	near skin	10	2–4	20	2–4	20[b]	2–4
	cortex-vascular ring	0	2	0	2–4	0	2–4
	central	0	0	0	0	0	0
10	all cut surfaces	0	0	0	0	0	0
100	all cut surfaces	0	0	0	0	0	0

[a] Continuity of tiers expressed as percent of total linear distance of cut surfaces within which tiers were adjacent and contiguous.

[b] Up to 90% continuity was found in skin and cortex area in one sample.

et al. (1962), such cell enlargement apparently has not been described previously for wound periderm inhibition by either chemical or irradiation treatment.

Table 1 summarizes results obtained with CIPC treatments and includes comparisons of inner and outer tissue zones as to the range of cells per tier and degree of continuity following different incubation periods. For practical purposes, numbers of cells per tier represent twice the number of cell divisions, each division of a phellogen cell in a plant parallel to the surface producing two cells, one of which remains cambial to divide again. As additional periderm cells are formed, the outermost mature and their walls suberize. Suberization also occurs independently in the walls of the originally cut cells and those immediately subjacent, and this often occurs before wound periderm is initiated. In all samples here, suberization was pronounced after 10 days of incubation, as revealed either by staining with safranin in prepared sections or with Sudan IV on fresh sections.

Sprouting was completely inhibited at levels of 10 ppm or more of CIPC in all of these samples incubated at 70–75°F and 88% relative humidity. All tubers receiving 1 ppm CIPC showed only a slight retardation of sprouting. In contrast to these results with tuber storage at room temperature, in earlier experiments inhibition of sprouting was complete in tubers receiving only 1–2 ppm CIPC prior to storage for 7 months at 50°F (Hendel, 1957).

DISCUSSION

Wound-healing potential varies in different tubers and within different tissue zones of the same tuber to a degree that could be significant to problems of storage and keeping quality. In comprehensive histological studies, Priestley and Woffenden (1923) compared 20 varieties as to efficiency of wound healing. Weak, or incomplete, wound periderm formation appears to be characteristic of some potatoes. Such variability may serve to explain some of the differences in results of different investigations on sprout inhibition, wound healing, and incidence of spoilage.

Relatively little histological detail has been published on effects of sprout-inhibiting treatments on wound periderm, despite much experimental investigation. Smith and Smart (1955) established a rating basis by which they compared suberization and wound healing with reference to bacterial decay. Waggoner (1955) reported the effects of irradiation on number of cell divisions occurring 3 days from wounding after 49–67 days following irradiation treatment, but he included no data on continuity of wound periderm. As indicated in present studies, there is a wide variation between tubers as to rate of phellogen cell divisions forming the wound periderm during the first few days after wounding.

Although the significance of wound healing for spoilage resistance has long been recognized, the relative importance of suberization and periderm continuity does not appear to have been fully understood by earlier workers. In more recent studies, Cunningham (1953) and Smith and Smart (1955, 1959) pointed out the importance of wound periderm continuity for rot resistance. Both continuity and depth of wound periderm (by average cell number) were compared in cut tubers by Bonde and Hyland (1960), who investigated the effects of agrimycin and captan treatments. The present studies also indicate the importance of wound periderm continuity, and further confirm other published reports that wound healing is inhibited by lower levels of chemical and irradiation treatments than those required to inhibit sprouting.

Various means of application of chemical sprout inhibitors have been investigated. Marth and Schults (1950) found that CIPC was effective when applied either as a dust or as a water emulsion. Sawyer and Dallyn (1957, 1961) found CIPC a more effective inhibitor than several other chemicals, and they obtained excellent results by vaporizing it as a dose of 0.25 g per bushel. Bishop and Schweers (1961) found that airplane application of malic hydrazide at 3 lb per acre effectively reduced shrinkage losses during in-ground and post-harvest storage of fall-grown potatoes.

Audia *et al.* (1962) obtained results very similar to those reported here. In their investigations, potato slices were dipped in different concentrations of CIPC solutions, then held at 60°F. They did not obtain complete inhibition of wound periderm even after dip in CIPC at 100 ppm, although only a trace of periderm development occurred after treatment at this concentration. Full comparison between vapor application and dip treatment with CIPC, as to inhibitory effect, however, would require analyses for residual CIPC present after different periods of storage.

The physiological diversity of tissues within the potato tuber has significance for wound healing. Cortical tissues underlying the skin possess greater healing potential than do the storage parenchyma cells well inward from the vascular ring, as described by Werner (1938). Sprout-inhibiting treatments that do not block initiation of wound periderm in the cortex completely inhibit healing of the deeper tissues. Superficial wounds, therefore, may pose less critical problems than deeper wounds, where failure to form a wound barrier could lead to losses from spoilage and impairment of processing quality. Histological evaluation of

healing of puncture wounds will be discussed in a later paper.

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Histological Analysis of Wound Healing in Potatoes Treated to Inhibit Sprouting II. Puncture Wounds and Fusarium Inoculates

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SUMMARY

Isopropyl-N-3-chlorophenyl carbamate (CIPC) applied as emulsion dips of 0.5, 1.0, and 2.0% concentration totally inhibited wound periderm formation by potato tubers. At these concentrations, CIPC prevented growth of *Fusarium sambucinum* inoculates except in a few special cases. Some fungal growth occurred in puncture wounds in which trapped air at the time of dip blocked CIPC penetration prior to inoculation.

Penetration of CIPC at 1.0 and 2.0% concentrations into coarse puncture wounds killed the tissues, and a rapidly spreading breakdown resembling bacterial soft rot then occurred. Smear preparations of the spoilage material revealed no microscopic evidence of microorganisms.

Catechol applied by dipping in solutions of 0.1 and 0.5% had no obvious effect either on wound periderm or fungal growth. Catechol toughened exposed wound surfaces but did not appear to increase suberization deep within puncture wounds.

Selected examples of within-tuber variations in wound healing and a brief survey of the histology of wound healing are also presented.

INTRODUCTION

Suppression of wound periderm formation on cut, exposed surfaces of potatoes by CIPC (isopropyl-*N*-3-carbamate) at levels lower than those required for sprout inhibition has been described (Reeve *et al.*, 1963). The earlier report included comparisons of wound periderm in different tissue zones of the tuber. Tissues internal to the xylem "ring" did not form wound periderm as rapidly as did the cortical tissue, and averaged fewer cells per tier by the time the wound periderm was continuous.

Although it is known that superficial wounds heal more rapidly than do deep punctures into the tuber (Werner, 1938), no recently published investigations have included detailed histological descriptions of these differences in relation to effects produced by sprout-inhibiting treatments.

To evaluate further the retardation of wound periderm formation by CIPC, tests were made on puncture wounds in whole potatoes and also upon the influence of CIPC upon germination of *Fusarium sambucinum* spores introduced into deep puncture wounds. Natural phenolics associated with the suberization process are known to accumulate in wound areas and have been thought to have a fungistatic role (Johnson and Schaal, 1957; Lee and LeTourneau, 1958; Simonds *et al.*, 1953). Therefore, the effects of catechol, both alone and combined with CIPC, were also investigated.

The results of these treatments are presented here, along with a brief survey of histological phenomena peculiar to wound healing.

MATERIALS AND METHODS

Wounding. Freshly harvested California-grown Russet Burbank potatoes were used. All tubers were washed in water, blotted, and allowed to dry 2–3 hr. Puncture wounds, ¾–1 in. deep, were made with a 20-penny nail or with a sharp knife, each alcohol-flamed before each puncture. In one

[a] Deceased.

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

lot of tubers, two wounds of each type were made at the bud end, at the middle, and at the stem end of each tuber. In addition, some tubers were sliced in half longitudinally.

Dips. CIPC and catechol were applied, both alone and in combination, by dipping the wounded tubers for 5 sec in agitated water emulsions. A commercial product containing about 42% of CIPC was used to prepare concentrations of 0.5, 1.0, and 2.0% CIPC. Control tubers (0 CIPC) were dipped in sterile water to provide comparable conditions of moisture exposure. Puncture wounds in which air obviously was entrapped, blocking immediate penetration of CIPC, were marked for future recognition by tiny notches adjacent to the puncture orifice.

Catechol was used in solution at concentrations of 0.1 and 0.5%. The experimental design was a full 3 × 4 factorial.

Inoculation and incubation. Another lot of tubers treated as described above were inoculated with *Fusarium sambucinum* after treatment. One drop of a water suspension containing the fungus spores was applied from a sterilized medicine dropper to each opening of the puncture wounds and at marked areas on the cut surfaces of halved tubers. Inoculated and uninoculated tubers were incubated at 70°F separately in 5-gal cans under conditions previously described (Reeve *et al.*, 1963), or with 8 or 10 tubers per container for each treatment.

Histological preparation. Samples for histological examination and general observations were removed from incubation after 3, 6, 9, and 18 or 20 days. Replicate blocks of tissue surrounding the wound areas were cut out and killed and fixed separately for 24–48 hr in two standard preparations. One preparation consisted of about 7% each of propionic acid and commercial formalin in 60% aqueous ethyl alcohol; the other was a freshly prepared aqueous mixture of 0.5% chromium trioxide, 8% acetic acid, and 10% commercial formalin (CRAF).

The tissue blocks were then dehydrated and embedded in paraffin (mp 52°C) according to standard procedure. Serial sections were microtomed at 20 μ thickness and fixed to microslides with Haupt's gelatin adhesive and 2% formalin solution.

After removal of paraffin the sections were stained to differentiate the hyphae of *Fusarium sambucinum*. Margolena's (1932) modification of Stoughton's (1930) technique was used, with slight alteration in which the saturated orange G and erythrosin solutions were used in a 1:1 mixture following staining in phenolthionin and light green. With proper timing in this schedule, the fungal hyphae were stained lavender, starch granules blue, suberized and lignified cell walls light blue-green, cell walls of healthy host tissue red-brown to orange, and those of unregenerated wound tissue (i.e., lacking wound periderm) a blue-purple.

RESULTS

Uninoculated tubers. Very little difference in potential for wound periderm formation was observed between bud ends, middles, and stem ends of control tubers incubated up to 20 days, whether the wounds were punctures or cut surfaces. In only a few instances did there appear to be a slightly more rapid initiation of wound periderm at the bud ends than at the middle and stem ends of the tubers; these cases were confined to the cortex of longitudinally-sliced tubers.

The various tissue zones of longitudinally-sliced tubers exhibited characteristic differences in potential for forming wound periderm. As previously described (Reeve *et al.*, 1963), wound periderm is initiated more rapidly in the cortical tissues underlying the skin than in more centrally located storage parenchyma. Cells of the "water core" or pith appeared to be the slowest to undergo wound periderm initiation.

These differences between tuber zones in rates of phellogen initiation and activity were found to be more pronounced in deep wounds made by knife punctures than on the exposed surfaces of longitudinally-sliced tubers. In addition, wound periderm formation was slower in the puncture wounds than on the cut surfaces of halved tubers (Table 1). In general, the wound periderm of tuber halves had developed nearly as much in 3 days at 70°F as had that of knife punctures in 6 days. A continuous, well-defined wound periderm was developed in the deeper tuber tissue zones of longitudinally-sliced tubers by 6 days. By 6 days, most knife punctures had opened slightly to about the depth of the vascular "ring." Wound periderm at this site still was not continuous (compare Figs. 1 and 2). In addition, wound periderm was not continuous in the deeper tissues penetrated by knife punctures even after 20 days of incubation (Table 1).

Irregular proliferations of cells also were observed in some of the deeper knife punctures. These proliferations produced "islands" of callus between which the typical wound periderm was weakly developed or confined to the initial divisions. Suberization was also weak in these more internal areas. Olufsen (1903) described similar proliferations of callus that were unsuberized, and considered that this resulted from excess moisture.

Such variable degrees of wound periderm formation occurred in deep puncture wounds made

Table 1. Wound periderm development of uninoculated controls: longitudinally sliced tubers and tubers wounded by knife punctures.

Type of wound	Tissue region	Wound periderm							
		Incubation, 3 days		Incubation, 6 days		Incubation, 9 days		Incubation, 20 days	
		Percent continuity [a]	Range of cells per tier	Percent continuity [a]	Range of cells per tier	Percent continuity [a]	Range of cells per tier	Percent continuity [a]	Range of cells per tier
longitudinally sliced	near skin	40	2–4	90	2–6	100	2–8	100	4–8
	cortex-vascular "ring"	50	2–6	100	4–8	100	4–10	100	6–12
	central [b]	30	2–4	100	2–6	100	4–8	100	4–10
knife puncture	near skin	20	2–4	60	2–4	100	2–8	100	4–8
	cortex-vascular "ring"	30	2–4	80	2–6	100	4–8	100	4–8
	central [b]	0	0	30	2–4	70	2–6 [c]	90	2–8 [c]

[a] Percent of total linear distance examined in which tiers of cells were adjacent and contiguous.
[b] Mainly internal phloem parenchyma.
[c] Does not include "islands" of callus formation.

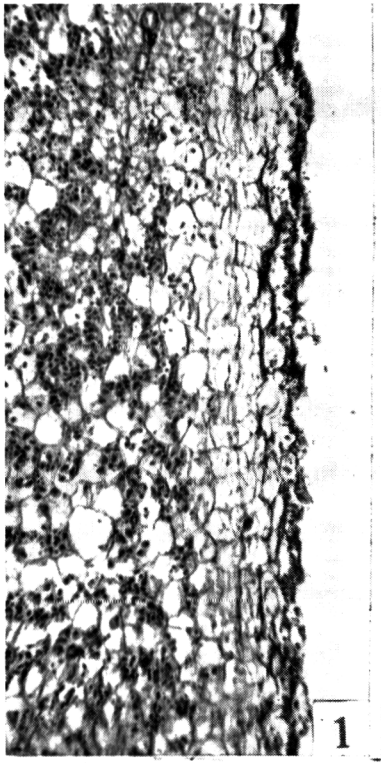
by a nail that no attempt was made to tabulate the results of observations. Suberization was not pronounced deep in these wounds; there was much debris of deeply-staining crushed cells and starch granules along the wound surface (Fig. 3). In addition, the punctures tended to be square in cross-sectional view and there often were irregular cracks extending into the tuber tissues. Wound periderm was very slow to form along these cracks, and some cracks showed no initiating cell divisions even after 20 days of incubation. Similar cracks, associated with wire puncture wounds, were described by Werner (1931).

Other variations in degree of wound periderm development occurred between different nail puncture wounds at comparable locations in the same tuber and also at opposite puncture sides at the same cross-sectional depth levels within individual punctures. In some nail punctures, the wound periderm on one side of the puncture was developed as well as on cut surfaces of longitudinally-halved tubers, while on the opposite side, it was less developed than in knife punctures for comparable times of incubation. In both types of puncture wounds, wound periderm initiation and development was more rapid at sites of vascular tissue strands (principally of internal phloem strands) than in adjacent storage parenchyma (Figs. 4, 5).

Uninoculated dipped tubers. No conclusive distinction in wound periderm formation was observed between untreated tubers and those receiving catechol dip but no CIPC. Simonds *et al.* (1953) found certain quinones more effective than catechol as precursory substrates for the suberization process. In the present studies, catechol appeared neither to alter wound periderm initiation, at concentrations used, nor to cause increased suberization within the deep puncture wounds. However, the wound skins formed on the cut, exposed surfaces which were both tougher and more deeply staining in tuber halves treated with catechol than in untreated tubers. Staining of freshly cut sections of these treated samples either with ferric chloride or with the nitrous acid reaction (Reeve, 1951) indicated that catechol had been either adsorbed or incorporated into the cell walls at the wound surfaces. No evidence could be found that the catechol had penetrated into puncture wounds to be either adsorbed or otherwise incorporated into the cell walls of the internal wound surfaces.

Wound periderm formation was blocked in all samples dipped in CIPC emulsions. Cell enlargement, concomitant with wound periderm inhibition, as previously described (Reeve *et al.*, 1963), was pronounced (Fig. 6).

Many of the puncture wounds opened wide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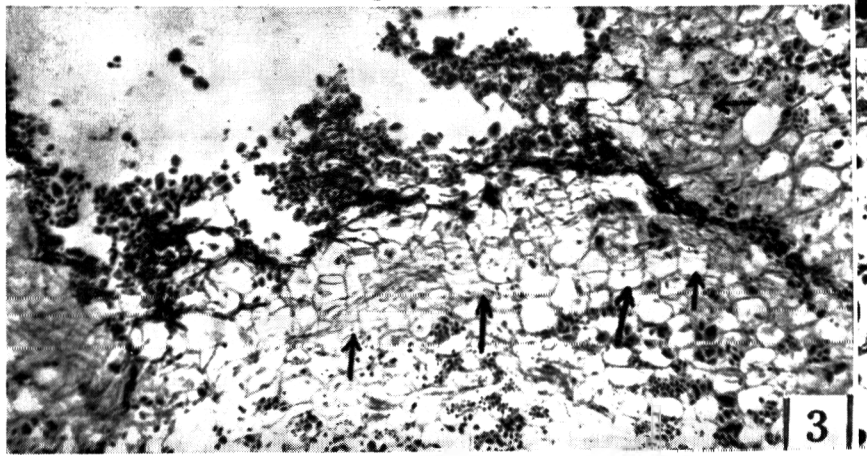
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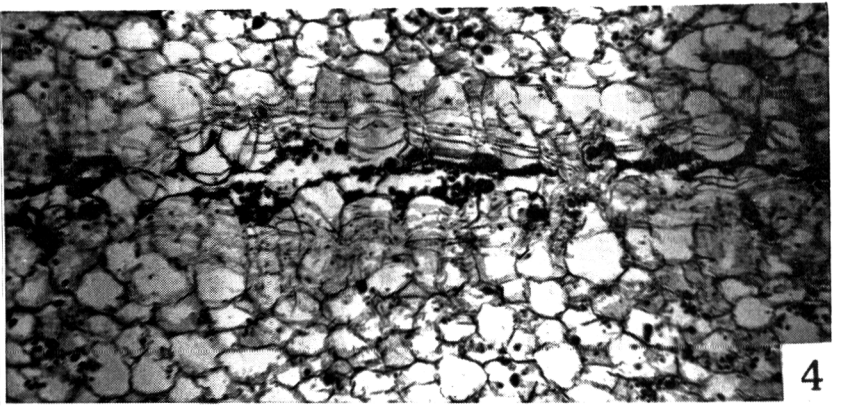
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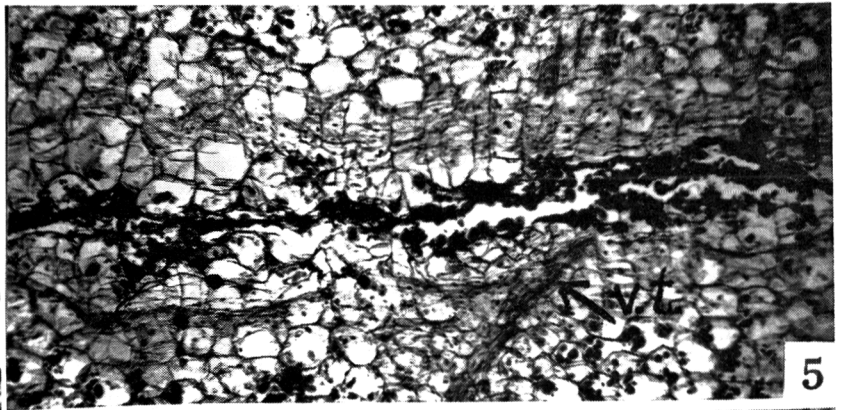
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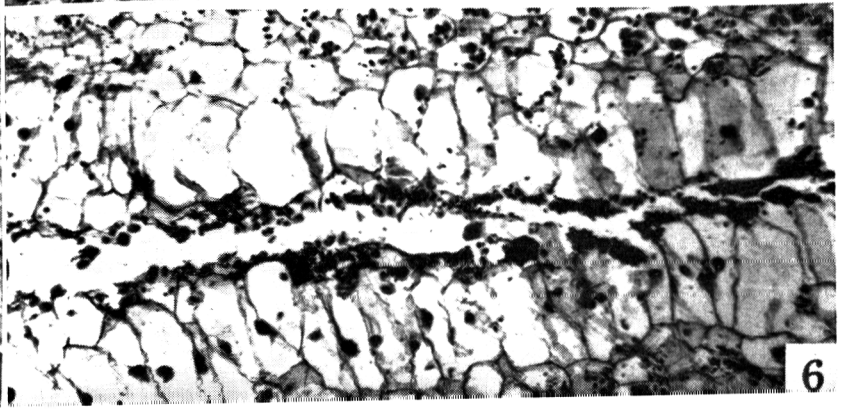
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during incubation, and those in tubers receiving CIPC treatments often became darkly discolored. It was not possible to obtain a complete histological series for each treatment. Most of the tubers receiving 1 and 2% CIPC dip spoiled rapidly after 6 days of incubation. It was evident that the CIPC had penetrated some of the puncture wounds of these tubers and killed the tissue. This was followed by an extensively spreading breakdown resembling bacterial soft rot. Such breakdown occurred within 2 or 3 weeks throughout most of the tuber tissues, and only in a few less extreme cases, in which the puncture wounds had opened wide in the first week of incubation, did the breakdown fail to occur and spread. Some tubers, appearing to be sound, consisted of a fairly firm shell of normal tissue enclosing a semi-liquid, evil-smelling, and discolored mass of decomposing cells. However, microscopic examinations of smear preparations failed to reveal any microorganisms. If not microbiological origin, it is possible that this breakdown was autolytic, i.e., a result of unrestricted enzymatic activity.

Inoculated tubers. Nearly all inoculated wounds in tubers receiving no CIPC showed profuse growth of *Fusarium sambucinum* after 3–6 days of incubation. Catechol treatments appeared to have little or no effect under these conditions. Fungal growth was most pronounced in the nail puncture wounds of untreated tubers, where the environment apparently was more favorable than on the exposed cut surfaces. Growth was much less in the knife punctures than in nail punctures after 3 days' incubation, but fungal growth was very pronounced in both types of punctures after 6 or more days of incubation. No evidence could be found of wound periderm initiation in tissues underlying the fungal penetration. In a few instances wound periderm had been initiated before fungal growth was profuse (Fig. 7), but the growing hyphae penetrated the tissue rapidly (Fig. 8), both along the middle lamellae of adjacent cells and through the primary pit fields of the cell walls (Figs. 9, 10).

A few of the inoculated punctures of tubers dipped in the 0.5% CIPC emulsion also showed an appreciable growth of the fungus. These few cases, however, were confined to samples incubated only 3 or 6 days and at wound sites known to contain entrapped air bubbles following dip in CIPC. Presumably, this entrapped air in the puncture had blocked penetration by CIPC, thus permitting germination of the spores. No fungal growth occurred in all other inoculated wounds of CIPC-treated samples.

DISCUSSION

These histological observations further verify phenomena known to investigators of problems in potato storage pathology and wound healing. Werner (1938) described differences in degree of wound periderm formation between cortical and inner tissues of tubers similar to those described here. His investigations of knife punctures ("radial cuts" into tubers) included an extensive survey of wound healing under a wide variety of conditions. However, in describing variations in healing in different tissues of the tuber, Werner used an older terminology. For example, "pericycle" is not considered to have morphological significance in more recent concepts in plant anatomy (Esau, 1953). Also, he apparently regards the "perimedullary zone" as being synonymous with "pith." In the stricter sense, "medulla" is "pith" or "water core" in the potato tuber, and, by etymology, "peri-

CAPTIONS FOR FIGURES ON OPPOSITE PAGE

Fig. 1. Wound periderm development of exposed cut surface of control tuber after 6 days of incubation. ×40.

Fig. 2. Discontinuous wound periderm development at surfaces of an internal knife cut, about ⅛-in. deep from the tuber skin of a control tuber after 6 days of incubation. ×40.

Fig. 3. Wound periderm development after 6 days of incubation in nail puncture of untreated tuber at ⅝- to ¾-in. depth from skin. Note discontinuity of wound periderm in crack extending on right from puncture cavity. ×40.

Fig. 4. Nearly continuous wound periderm at about ¾-in. depth in knife puncture in an untreated tuber after 9 days of incubation. Cut extends laterally through the middle of photo; note irregularity of tier formation. ×40.

Fig. 5. Same as Fig. 4 except at a region through a vascular trace (V.T.). ×40.

Fig. 6. Cell enlargement deep in a knife cut of a tuber dipped in 0.5% CIPC emulsion. ×40.

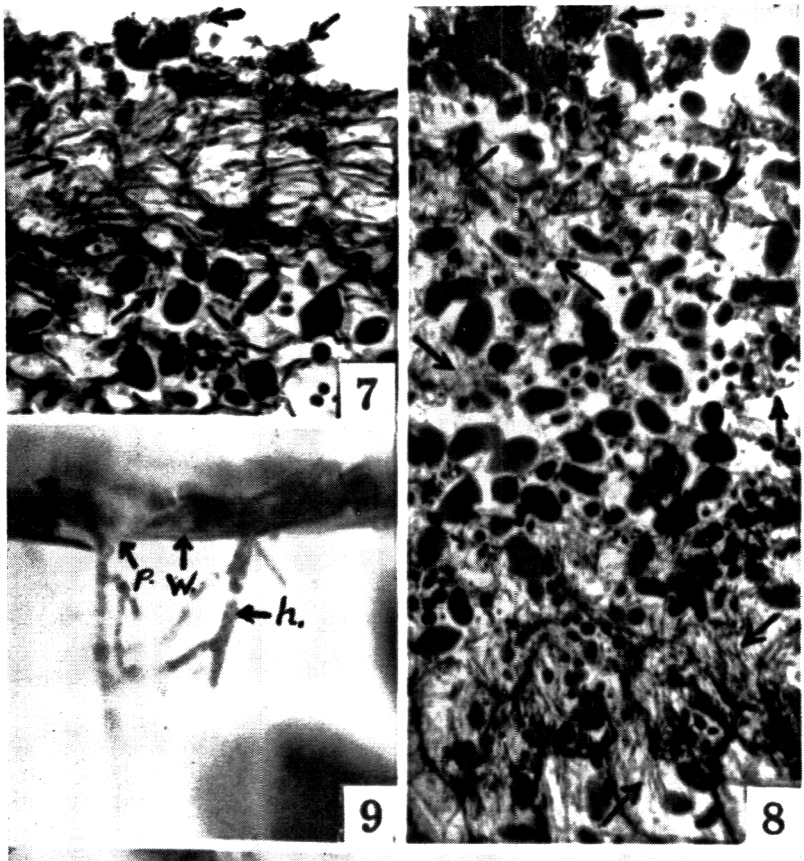

Fig. 7. Penetration of *Fusarium* hyphae through wound periderm initials and young tiers at wound surface of a knife puncture and ½- to ¾-in. depth from tuber skin; incubation time 6 days. Arrows at surface indicate clusters of germinating spores; hyphae in tissue show as fine lines. ×100.

Fig. 8. Deeper penetration of *Fusarium* hyphae after 6 days of incubation. ×100.

Figs. 9 and 10. *Fusarium* hyphae penetration of cell walls: p, primary wall pit, w, cell wall, h, hyphae. ×400.

medullary" tissue would be the internal phloem and its associated storage parenchyma, located inwardly from the xylem of the vascular "ring" (Artschwager, 1924).

The slower and sometimes erratic healing that may occur in puncture wounds suggests that such wounding poses more critical problems than do shallow wounds in storage of potatoes for processing. Spores of microorganisms introduced at the time of puncture have ample opportunity to germinate and infect internal tissues before a wound barrier is well initiated. Hence, fungicidal sprout inhibitors should be applied as soon as possible. CIPC, even at the fungicidal concentrations used here, did not always prevent fungal growth in deeper and protected punctures. In addition, penetration of such an agent as a dip emulsion of CIPC can kill the internal tissue and

render it more susceptible to either autolytic or microbiological breakdown.

The importance of adequate wound healing to keeping qualities of stored potatoes has been well demonstrated by other investigations. Cunningham (1953), Smith (1961), and Smith and Smart (1955, 1959) found that microorganisms can penetrate the wound surface when wound periderm is completely formed, even though suberization of the exposed cell walls may be continuous. Hooker and Duncan (1959) investigated the influence of irradiation on storage rot, using doses from 5,000 rep upward, and found that normal wound healing was delayed and that susceptibility to rot increased roughly in proportion to increased irradiation. The importance of wound healing to reduction of storage rot likewise has been demonstrated by Duncan *et al.* (1959) and Waggoner (1955).

Certain naturally occurring phenolics accumulate in wound areas; some are associated with the suberization process as precursors of suberin. Several workers have suggested that the fungistatic properties of these substances contribute to disease resistance (Johnson and Schaal, 1957; Lee and LeTourneau, 1958; Simonds *et al.*, 1953). Although possible use of phenolics to enhance suberization is of academic interest, economic and other considerations at present limit their practical application.

Optimum conditions of temperature and relative humidity, and availability of oxygen, for the formation of a continuous wound barrier have been defined by Artschwager (1927). The times and temperatures exceed those of some of the more recently published recommendations for conditioning of potatoes prior to sprout inhibitor application. Because deep puncture wounds heal slowly, they present a more critical problem than do shallow, rapidly healing wounds.

The objectives here and in the preceding paper have been confined to a survey of the histological phenomena involved in wound healing. Recommendations for treatment and storage of potatoes, however, cannot be based solely upon histological considerations. The variations in potential for wound healing of different tissues within single tubers and between different tubers according to variety, age, and other environmental history render over-all evaluations difficult.

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Studies on the Emulsifying Properties of Some Intracellular Beef Muscle Proteins[a, b]

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SUMMARY

The emulsifying capacity curves for actin, myosin, actomyosin, and sarcoplasmic proteins were determined under various conditions. The proteins were ranked from greatest emulsifying capacity to least as follows: actin in the absence of salt, myosin, actomyosin, sarcoplasmic proteins (water extracted), and actin in 0.3*M* salt.

Myosin and actomyosin produced emulsions with superior stability; however, at the pH of normal fresh meat (5.6–5.8), the sarcoplasmic fraction produced the most stable emulsions. Actin produced very stable emulsions under all conditions. The amount of protein utilized in the formation of an interface appeared to be related to the stability of an emulsion. NPN compounds were found to have no role in emulsion formation.

INTRODUCTION

Hansen (1960) and Swift *et al.* (1961) have established that fat incorporated into a sausage product is dispersed in small droplets and enveloped with a layer of protein material, producing essentially an oil-in-water emulsion. Swift *et al.* (1961) and Swift and Sulzbacher (1963) utilized meat slurries and crude muscle extracts to study fat-emulsifying properties in model systems. The present study investigated the emulsion-forming and stabilizing properties of individual purified intracellular muscle protein fractions.

EXPERIMENTAL

Protein isolation. The sarcoplasmic proteins and actomyosin were isolated from frozen longissimus dorsi muscles of Good- or Choice-grade steers. All protein extractions were performed at 4°C unless otherwise stated. Extracts were made of sarcoplasmic proteins (actually a complex mixture of similar proteins), using either 70 ml water or a phosphate buffer (pH 7.6, $\mu = 0.05$) and 5 g muscle. The phosphate buffer was used in order to extract the globulin X fraction along with the other sarcoplasmic proteins.

Actomyosin was prepared by the method of Szent-Györgyi (1951). A predominance of actomyosin in the extract was verified with an ATP sensitivity test involving observed drop in viscosity upon the addition of a small amount of ATP.

Myosin was prepared according to the method of Mommaerts and Parrish (1951), and actin was prepared as outlined by Tsao and Bailey (1953). Actin and myosin were prepared from the same muscle sample, i.e., the longissimus dorsi muscles of young calves. An attempt was made to extract these proteins from the neck muscle of yearling steers, but this source proved unsatisfactory for myosin. It was concluded that too much unseparable fat was contained in the neck muscles, and when this lipid material was carried through the procedure it caused a large amount of surface denaturation of the myosin preparation. Longissimus dorsi muscle samples were generally obtained from three-month-old calves within three minutes of death. All separable fat and connective tissue were removed from the sample, which was ground through a 2-mm plate. The buffer described by Mommaerts and Parrish (1951) was used to extract 400 g of ground muscle. The residue from this extraction was pressed as dry as possible and retained for the preparation of actin acetone powder. This procedure was performed at room temperature.

Mommaerts and Parrish (1951) stated that preparations of rabbit myosin were stable for about one week. Calf myosin obtained with their method, however, began to show evidence of denaturation in 24–48 hours as indicated by the appearance of insoluble threads in the solution. It was found that the myosin preparation was more stable when stored in a salt solution of high ionic strength, i.e., 1*M* KCl.

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Actin was extracted from the above-mentioned acetone powder with approximately 25 volumes of cold water that contained 20 mg ATP/100 ml. The protein content of this solution was concentrated to the necessary level by pervaporation.

A sample of calf myosin in 1M KCl (protein concentration, 1 percent) was subjected to ultracentrifugation in a Model E Spinco ultracentrifuge at 59,780 rpm at 0°C. A large, very sharp myosin peak with an S_{20} of 3.14 indicated that the preparation was myosin with a sufficient degree of purity for the preparation of emulsions.

The amount of active actin in the actin preparation was determined to be 34% by allowing the actin to react with the purified myosin and measuring the amount of unreacted actin present. Actin data were calculated from total actin, both active and inactive.

pH adjustment of protein solutions. Emulsion stability and emulsifying capacity of proteins were studied in the acid, alkaline, and neutral pH ranges. The pH of all protein solutions as extracted was near neutrality, except the sarcoplasmic fraction, which was approximately pH 5.5. The pH was increased from neutrality by adding solid Na_2CO_3 or $NaHCO_3$, and decreased by adding KH_2PO_4. The pH of the sarcoplasmic protein solution was adjusted to neutrality from 5.5 by adding solid K_2HPO_4 and KH_2PO_4 in a predetermined ratio. Na_2CO_3 was used to adjust the sarcoplasmic protein solution to an alkaline pH. Calculated amounts of the above materials were added to produce an increase in ionic strength of 0.05. The ionic strength of solutions with unchanged pH, i.e., those studied as extracted, was increased by 0.05 with KCl in order to remove the variable of ionic strength from the pH experiments.

Emulsion preparation and evaluation. Emulsions were prepared in a manner similar to that described by Swift *et al.* (1961). The principal difference between the two methods was the stirrer speed: 1750 rpm. vs. their stirrer speed of 13,000 rpm. A Lightnin Model L stirrer was used, equipped with a three-bladed propeller with a diameter of 5 cm. The amount of soybean oil added to the emulsion is reported in g, and emulsifying capacity is expressed as g of oil emulsified/mg of protein nitrogen in the solution.

After the emulsion was broken by adding an amount of oil that exceeded the emulsifying capacity of the protein, it was centrifuged as described by Swift *et al.* (1961) and the aqueous layer analyzed for nitrogen. The amount of protein nitrogen removed from the aqueous phase during emulsification was divided by the amount of protein nitrogen originally present, and the resulting percentage value is reported as a supplementary measure of emulsion stability.

The relative stability of emulsions prepared from the various protein preparations was determined by storing at room temperature and noting the degree of fat separation and loss of white color as the emulsions aged. Emulsions for stability tests were prepared with 25 ml of protein solution (concentration 0.5 mg of protein nitrogen/ml) and 200 g of oil. When the protein in question would not emulsify 200 g of oil, the amount of oil was reduced to a suitable level. All emulsions were white immediately after preparation, and their stability determined by noting the time when noticeable fat separation had occurred.

RESULTS AND DISCUSSION

The emulsifying capacity curves (Figs. 1, 2) indicated that the emulsifying capacity of the sarcoplasmic fraction was higher when no salt was added. When protein concentration was plotted against quantity of oil emulsified, a marked difference was ob-

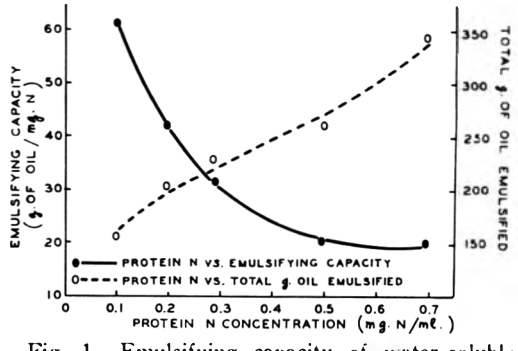

Fig. 1. Emulsifying capacity of water-soluble sarcoplasmic protein and g of fat emulsified vs. concentration of protein in aqueous phase (no added salt, pH 5.5).

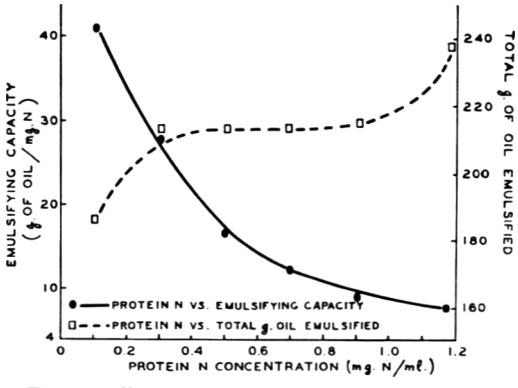

Fig. 2. Emulsifying capacity of water-soluble sarcoplasmic protein and g of oil emulsified vs. concentration of protein N in aqueous phase (protein in 0.6M KCl, pH 5.6).

served. The curve for the water solution was practically linear, whereas a sigmoid type of curve was obtained for the salt-containing protein solution. In the presence of 0.6*M* KCl the total g of oil emulsified remained constant for a range in protein concentration from 0.3 mg nitrogen/ml to 1.0 mg nitrogen/ml. In this range, approximately 210 g of oil were emulsified, regardless of protein concentration.

Swift *et al.* (1961) reported that the water-soluble protein fraction in the absence of salt had no marked capacity in stabilizing an emulsion, and Hansen (1960), using histological techniques, found that the water-soluble protein fraction was not observed at the oil-water interface of a sausage emulsion. However, the data in Fig. 1 tend to contradict these findings. In stability studies discussed later, it was found that emulsions prepared with the water-soluble proteins in salt solution were considerably more stable at normal pH's (5.5–5.7) than emulsions stabilized with this protein fraction in the absence of salt.

The behavior of myosin and actomyosin in emulsion systems could not be studied at low ionic strength or in pure water, since they were insoluble under these conditions. However, actin, once extracted from muscle tissue, was completely soluble in water. Fig. 3 presents curves representing the emulsifying capacity of myosin in 0.3*M* KCl and actin in both 0.3*M* KCl and water. Differences in the emulsifying capacities of these fractions are clearly evident. The presence of salt greatly depressed the effectiveness of actin. The emulsifying capacity of myosin was intermediate as compared to the actin solutions tested above. This relationship was always observed, regardless of protein concentration.

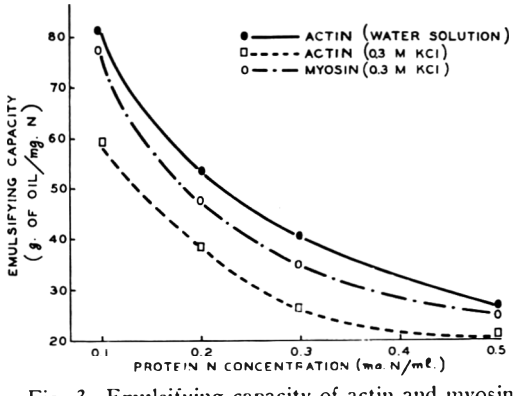

Fig. 3. Emulsifying capacity of actin and myosin vs. concentration of protein N in aqueous phase.

Fig. 4 shows the effect of ionic strength on emulsifying capacity of actomyosin. The

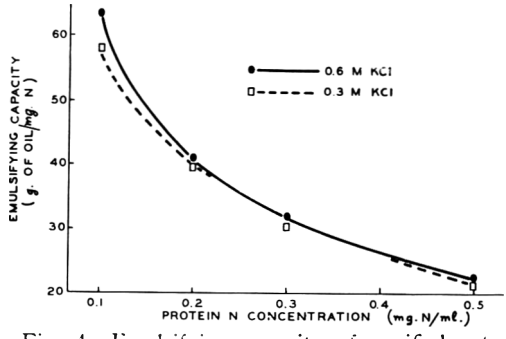

Fig. 4. Emulsifying capacity of purified actomyosin vs. concentration of protein N in aqueous phase (protein in 0.6*M* + 0.3*M* KCl).

similarity of the curves for protein solutions in 0.3*M* KCl and 0.6*M* KCl indicate that ionic strength, at least in this range, had no apparent influence on the emulsifying capacity of actomyosin. The shape of the above curves was characteristic of emulsifying-capacity curves for the meat proteins studied in this experiment. Without exception, the proteins studied exhibited a greater emulsifying capacity/unit of protein as the protein concentration was reduced.

About 7% of the nitrogen in muscle is non-protein nitrogen. To investigate the role of these compounds in emulsion systems, the sarcoplasmic fraction extracted with phosphate buffer was subjected to dialysis, which removed practically all of the non-protein nitrogen and caused precipitation of the globulin X fraction. Fig. 5 gives curves of emulsifying capacity of this fraction before and after dialysis. From these curves it appeared that neither globulin X nor the non-protein components were involved in emulsion formation. A noticeable difference was observed only at the lowest concentration, where the emulsifying capacity of the non-dialyzed fraction was the highest. A water-extracted sarcoplasmic fraction was also dialyzed to remove non-protein nitrogen. A complete curve was not

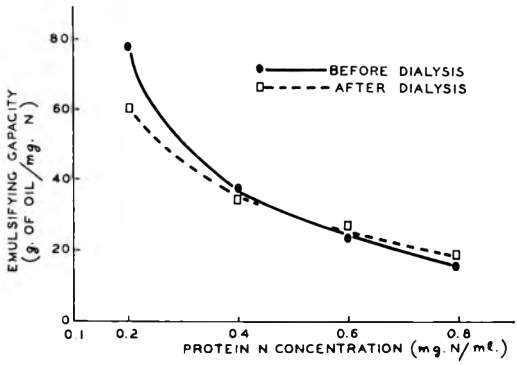

Fig. 5. Emulsifying capacity of sarcoplasmic proteins (buffer extracted, $\mu = 0.5$, pH = 7.6) before and after dialysis vs. concentration of protein N in aqueous phase.

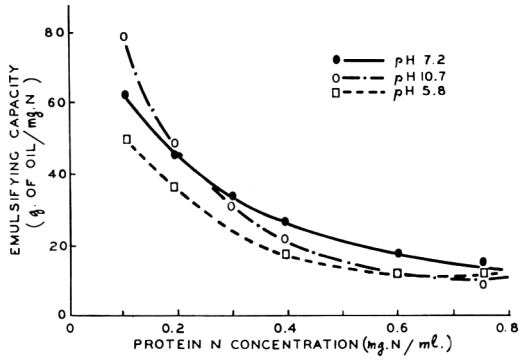

Fig. 7. Emulsifying capacity of purified actomyosin vs. concentration of protein N in aqueous phase as affected by pH ($\mu = 0.65$).

determined in this case, but approximately identical emulsifying capacities for dialyzed and non-dialyzed protein solutions at several protein concentrations indicated that non-protein compounds had no effect in forming emulsions. These results are in agreement with those reported by Swift *et al.* (1961).

The effect of pH on emulsifying capacity was studied with the actomyosin and sarcoplasmic fractions (Figs. 6, 7). The pH effect in this regard seemed to be quite small in that the only noticeable difference due to pH occurred in the case of actomyosin (Fig. 7), when the emulsifying capacity was lowered slightly at low pH. At pH 5.8, actomyosin is approaching its isoelectric point, which probably had some effect on its ability to form at an interface.

Fig. 6 presents data obtained from pH studies involving two kinds of oil (soybean and cottonseed). At lower protein concentrations, apparent differences were observed

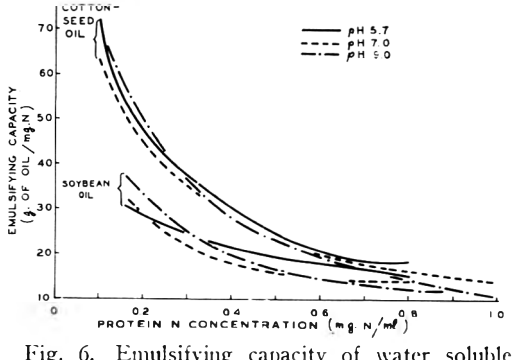

Fig. 6. Emulsifying capacity of water soluble sarcoplasm proteins vs. protein N concentration of aqueous phase as affected by pH and kind of oil.

between the two oils. However, the relative effect of pH for each oil did not differ, indicating that results obtained from one oil could be applied to another oil.

Table 1 gives information on the stability of emulsions prepared from the various protein fractions at various pH's and ionic strengths. Actomyosin and myosin were studied at only one ionic strength, 0.35. The low emulsifying capacity of actin, myosin, and actomyosin at low pH necessitated a reduction in the amount of oil added. These proteins produced very unstable emulsions at low pH. Actomyosin and myosin at near-neutral pH's produced very similar emulsions, which were superior to emulsions stabilized by any other proteins at any pH or ionic strength. Myosin and actomyosin also produced quite stable emulsions at alkaline pH's, although these were not as stable as those at or near neutrality.

Actin produced emulsions with extremely low stability at all pH's and ionic strengths studied. The possibility remains that actin in the fresh muscle may act differently from the actin utilized in this experiment. It is recognized that the preparation of an acetone powder may have caused appreciable changes in the proteins; however, more than one-third of the actin obtained in this manner was of the active type, i.e., it combined chemically with myosin.

A great deal of variation was observed in the stability behavior of emulsions prepared from the sarcoplasmic proteins. At low pH, i.e., 5.5 (approximately the pH of post-rigor fresh meat), and in the pres-

Table 1. Characteristics of emulsions prepared with different protein fractions under varying conditions.

Type of protein (conc. = 0.5 mg. N/ml)	pH	Solvent	g of soybean oil	Emulsion characteristics			
				Texture	Color [a]	Viscosity	Time stable
Actomyosin	5.4	0.3*M* KCl 0.05*M* KH_2PO_4	160	coarse	yellow	intermediate	7 days
Actomyosin	6.7	0.35*M* KCl	200	smooth	white	very high	>3 weeks
Actomyosin	8.0	0.3*M* KCl 0.05*M* $NaHCO_3$	160	smooth	white	very high	>3 weeks
Actomyosin	10.7	0.3*M* KCl 0.05*M* $NaHCO_3$	200	smooth	white	very high	2 weeks
Sarcoplasmic	5.5	Water	200	smooth	yellow	low	3 days
Sarcoplasmic	5.6	0.35*M* KCl	200	smooth	yellow	low	2 weeks
Sarcoplasmic	6.7	0.30*M* KCl 0.0157*M* K_2HPO_4 0.0035*M* KH_2PO_4	160	grainy	yellow	low	12 hours
Sarcoplasmic	7.0	0.0157*M* K_2HPO_4 0.0035*M* KH_2PO_4	200	fairly smooth	yellow	low	2 days
Sarcoplasmic	9.0	0.017*M* Na_2CO_3	200	smooth	white	intermediate	10 days
Sarcoplasmic	10.0	0.30*M* KCl 0.017*M* K_2CO_3	125	fairly smooth	yellow	very low	3 hours
Actin	5.7	0.05*M* KH_2PO_4	150	very smooth	yellow	low	<36 hours
Actin	7.2	Water	200	very smooth	yellow	low	<36 hours
Actin	7.2	0.35*M* KCl	200	very smooth	yellow	low	<36 hours
Actin	8.0	0.05*M* $NaHCO_3$	200	very smooth	yellow	low	<36 hours
Actin	8.0	0.05*M* $NaHCO_3$ 0.30*M* KCl	200	very smooth	yellow	low	<36 hours
Myosin	5.3	0.30*M* KCl 0.05*M* KH_2PO_4	125	coarse	yellow	intermediate	3 days
Myosin	5.8	0.35*M* KCl	200	smooth	white	very high	>4 weeks
Myosin	8.0	0.30*M* KCl 0.05*M* $NaHCO_3$	200	smooth	white	high	2 weeks
Crude extract	6.7	0.30*M* KCl 0.003*M* Na_2CO_3 0.01*M* $NaHCO_3$ 0.05*M* KH_2PO_4	200	smooth	yellow	intermediate	24 hours
Crude extract	7.6	0.30*M* KCl 0.003*M* Na_2CO_3 0.01*M* $NaHCO_3$	200	smooth	yellow	intermediate	24 hours

[a] Characterized within 24 hours of emulsion preparation.

ence of 0.35*M* KCl, the sarcoplasmic fraction was superior to any other fraction in emulsion stability. If no additional salt was present at pH 5.5, the emulsion broke in about two or three days, which was very similar to results for emulsions prepared with sarcoplasmic protein solutions at pH 7.0 and at ionic strength 0.05. With these protein solutions at pH 9.0 and ionic strength 0.05, emulsions had more desirable qualities and were stable for about 10 days. Sarcoplasmic proteins at alkaline pH's and ionic strengths of 0.35 produced very unstable emulsions, breaking in a few hours. Emulsions prepared with crude protein extracts (containing primarily sarcoplasmic and actomyosin proteins in approximately equal proportions) were similar in stability to emulsions prepared with sarcoplasmic proteins.

Table 2. Percent of total nitrogen involved in the formation of an interface during emulsification under varying conditions and for various proteins.

Type of protein	pH	Ionic strength	% of total nitrogen involved in formation of an interface
Actomyosin	5.8	0.65	91.5
Actomyosin	7.2	0.65	88.7
Actomyosin	10.7	0.65	86.3
Actin	7.6	0.30	61.6
Actin	7.6	0.0	41.0
Myosin	6.8	0.30	90.0
Sarcoplasmic	5.7	0.0	59.8
Sarcoplasmic	7.0	0.05	27.5
Sarcoplasmic	9.0	0.05	21.6

Actomyosin and myosin in their native states are probably completely utilized in the interface of oil-in-water emulsions. The protein nitrogen recovered in the aqueous phase of a broken emulsion that had been stabilized by actomyosin or myosin, probably consisted of previously denatured actomyosin and myosin and contaminating protein of sarcoplasmic origin. Since the emulsions were prepared at room temperature, some of the myosin and actomyosin, which are extremely heat labile, may have been partially denatured before emulsification began, and their interfacial activity thereby reduced.

Data on the amount of protein nitrogen, which retained its solubility in the aqueous phase after maximum emulsifying capacity was attained, furnished additional information on the activity of the muscle proteins in emulsion systems (Table 2). The pH effect was investigated in the case of actomyosin and the sarcoplasmic fractions. There appeared to be a definite pH effect in the sarcoplasmic fraction, but only a small one in the actomyosin fraction. The amount of sarcoplasmic protein accumulating at the interface was twice as great at the low pH (5.7) as at the higher pH's. This was reflected in the greater stability observed in emulsions prepared with sarcoplasmic proteins at low pH. At the neutral and alkaline pH's the sarcoplasmic fraction showed a great deal of variation between duplicates; this was not observed with any of the other protein preparations studied. The amount of actin recovered from broken emulsions was in the same range as the amount of sarcoplasmic protein recovered. This was also reflected in the poor stability qualities of emulsions prepared with actin.

The important observation in Table 2 is the small amounts of protein recovered in the aqueous phase of actomyosin and myosin stabilized emulsions in relation to the actin and sarcoplasmic fractions. It appears that the amount of protein denatured at the oil-water interface is directly related to the stability of the resulting emulsion.

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Effect of Pre-Slaughter Treatments on the Chemical Composition of Various Beef Tissues[a]

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SUMMARY

Twenty-four Hereford steers were used to determine the effect of periodic electrical stimulation and limited feeding prior to slaughter on the chemical composition of the liver and certain muscles, which included the longissimus dorsi, psoas major and quadriceps femoris. Periodic electrical stimulation and limited feeding significantly increased the glycogen concentration of the liver. Periodic electrical stimulation significantly decreased the glycogen concentration in all three muscles studied. Limited feeding significantly decreased the glycogen concentration in the longissimus dorsi and quadriceps femoris muscles. Periodic electrical stimulation and limited feeding significantly decreased the lactic acid concentration in the psoas major and quadriceps femoris muscles. These treatments also produced small changes in all chemical components except sodium, in either the liver or the various muscles studied.

INTRODUCTION

Lewis *et al.* (1961, 1962a) have shown that stress from periodic electrical stimulation prior to slaughter significantly decreased the ash, glycogen, lactic acid, and organic phosphorus concentration and increased the protein concentration in certain muscles of swine. Briskey *et al.* (1959) have reported that exercise immediately prior to slaughter decreased the glycogen concentration in samples of the gluteus medius muscles of swine taken 40 minutes after slaughter. They also reported no significant effect of exercise immediately prior to slaughter on the glycogen, sodium, potassium and moisture concentration in samples of this muscle taken 24 hours after slaughter. Hall *et al.* (1961) reported that pigs that were fatigued from transportation to the slaughter plant contained less muscle glycogen than rested pigs. The purpose of this experiment was to determine if the effect of stress from periodic electrical stimulation and limited feeding prior to slaughter similarly influenced the chemical composition of various beef tissues.

EXPERIMENTAL

After an 18-hour shrink, 12 good and 12 choice grade Hereford steers were grouped according to weight into six groups. Weight differences were less than 75 pounds within each group. The animals within each group were then allotted at random to four treatments in a 2 × 2 factorial experiment. Lots 1 and 2 were full-fed a practical fattening ration which they had received previously for 120 days while lots 3 and 4 were fed only two pounds of hay per head per day during the week just prior to slaughter. For 24 hours immediately prior to slaughter, lots 2 and 4 were subjected to stress from periodic electrical stimulation while lots 1 and 3 were allowed to rest without feed and water. Periodic electrical stimulation consisted of 3 or 4 electrical shocks from an electric "hot shot" every 20 minutes for 24 hours.

Samples of the liver were obtained for chemical analysis 24 hours after slaughter and samples of the longissimus dorsi, psoas major and quadriceps femoris muscles were obtained for chemical analysis 14 days after storage in a 37–38°F chill room. The samples were taken at the times indicated above because these tissues are distributed and sold to the consumer after these storage times. Therefore, any changes in the chemical composition caused by pre-slaughter treatment might help explain any changes in the eating quality of these tissues. The following areas of the tissues were taken for chemical analysis: longissimus dorsi, the muscle between the 12th rib and the second lumbar vertebra; psoas major, the entire muscle appearing in the sirloin; quadriceps femoris, the entire muscle appearing in the round; and liver, a three-inch center section in the omasal area. All

[a] Published with the approval of the Director of the Arkansas Agricultural Experiment Station.

samples were ground in a Waring blender and frozen until moisture determinations could be made. These samples were then freeze-dried, ground again in a Waring blender and stored in glass bottles until the other chemical analyses were determined.

Moisture, protein, ash, and fat were analyzed by accepted methods of AOAC (1960). Glycogen was extracted with 30% potassium hydroxide and precipitated by adding 1.2 volumes of 95% ethyl alcohol as described by Pfluger (1905). The resulting precipitate was analyzed photometrically by the anthrone method of Colvin *et al.* (1961). Lactic acid was determined by the method of Barker and Summerson (1941). Inorganic phosphorus, sugar phosphorus, total phosphorus and non-protein nitrogen were analyzed by methods described by Hawk *et al.* (1954). Sugar phosphorus was designated as that amount of organic phosphorus extracted by cold (4°C) 10% trichloroacetic acid. This extraction was carried out in a cold room at 3–4°C. Meat samples were ashed by the method of Toth *et al.* (1948). Sodium, potassium, calcium, and magnesium were determined on a Beckman DU flame spectrophotometer with an electronic power-supply attachment (Beckman, 1957; Close *et al.*, 1953). Corrections were made for the interference of potassium on the magnesium determination, and for the interference of phosphate on the calcium determination, by adding to the standards the amount of these interfering materials present in the samples. All chemical components are reported as averages of four determinations on each sample. Chemical determinations were made at the same time on each weight group in the 2×2 factorial experiment.

Moisture was expressed on a fat-free, carbohydrate-free basis; lactic acid and glycogen on a fat-free, dry matter basis; protein, ash, inorganic phosphorus, sugar phosphorus, total phosphorus, potassium, sodium, calcium, magnesium and non-protein nitrogen on a fat-free, carbohydrate-free, dry matter basis; and fat on a fresh weight basis. The chemical components were expressed as above to endeavor to remove the effect of differences in fat, moisture and carbohydrate upon the other chemical components studied. When chemical components are expressed on a concentration basis, a change in one constituent automatically causes a change in the other. Unpublished results have indicated that periodic electrical stimulation significantly increased the concentration of moisture in muscles of swine when the moisture was expressed on a fat-free basis; but there was no significant effect on the tissue moisture when the moisture was expressed on a fat-free, carbohydrate-free basis. Fat was determined mainly to show that the animals in each treatment group were of the same carcass grade.

Statements regarding significant differences are based on analysis of variance in which all percentages were transformed to the arcsin before analysis. All differences discussed were significant differences ($P < .05$ or $< .01$).

Table 1. Effect of stress and limited feeding prior to slaughter on the chemical composition of the liver and certain beef muscles (av. of six animals)

	Full ration		Limited ration		Significant variation due to:		
	Not stimulated	Stimulated	Not stimulated	Stimulated	Limited	Stress	Interaction
Moisture, % [a]							
Liver	75.7	76.4	77.5	78.9	**	**	
Longissimus dorsi	76.9	76.4	77.7	77.2	**	*	
Psoas major	78.1	78.0	78.9	78.8	**		
Quadriceps femoris	78.0	77.5	78.5	77.8	*	**	
Protein, % [b]							
Liver	93.7	93.6	93.5	93.2	**	*	
Longissimus dorsi	95.2	95.1	95.4	95.3			
Psoas major	94.4	94.9	94.9	94.9			
Quadriceps femoris	94.9	95.0	95.0	95.1			
Fat, % [c]							
Liver	1.1	1.8	2.5	2.9	*		
Longissimus dorsi	1.9	2.1	1.9	2.9			
Psoas major	4.0	3.6	3.7	4.3			
Quadriceps femoris	2.1	2.3	1.9	2.5			
Ash, % [b]							
Liver	6.3	6.4	6.5	6.8	**	*	
Longissimus dorsi	4.8	4.9	4.6	4.7			

	Full ration		Limited ration		Significant variation due to:		
	Not stimulated	Stimulated	Not stimulated	Stimulated	Limited	Stress	Interaction
Psoas major	5.6	5.1	5.1	5.1			
Quadriceps femoris	5.1	5.0	5.0	4.9			
Glycogen, mg/g [d]							
Liver	24.70	45.39	67.98	149.52	**	**	*
Longissimus dorsi	9.39	1.99	3.89	0.57	*	**	
Psoas major	15.90	0.88	11.16	0.13		**	
Quadriceps femoris	16.58	0.80	7.21	0.18	**	**	
Lactic acid, mg/g[d]							
Liver	7.56	7.72	7.48	7.63			
Longissimus dorsi	34.97	34.71	34.24	32.07			
Psoas major	32.78	29.14	29.79	22.89	*	**	
Quadriceps femoris	34.27	32.70	33.13	25.79	**	**	*
Inorganic phosphorus, mg/g [b]							
Liver	3.93	4.10	4.53	4.29	**		
Longissimus dorsi	4.48	4.98	4.73	5.41		*	
Psoas major	5.05	5.71	5.54	6.08	*	**	
Quadriceps femoris	5.51	6.19	5.65	6.41		**	
Sugar phosphorus, mg/g [b]							
Liver	2.33	2.38	2.15	2.32			
Longissimus dorsi	1.54	1.26	1.59	0.84		*	
Psoas major	1.13	0.49	0.65	0.59			
Quadriceps femoris	1.15	0.89	0.97	0.45	*	*	
Total phosphorus, mg/g[b]							
Liver	15.66	15.91	16.30	17.18	*		
Longissimus dorsi	8.81	8.86	8.86	8.66			
Psoas major	9.28	9.54	9.24	9.33			
Quadriceps femoris	9.05	9.29	8.84	8.66	**		
Sodium, mg/g [b]							
Liver	2.53	2.38	2.27	2.39			
Longissimus dorsi	2.14	2.09	2.00	1.99			
Psoas major	2.12	2.31	2.26	2.37			
Quadriceps femoris	1.88	1.85	1.88	2.11			
Potassium, mg/g [b]							
Liver	13.77	14.45	14.60	16.64	*	*	
Longissimus dorsi	16.13	15.59	15.89	15.69			
Psoas major	16.24	16.29	16.01	16.35			
Quadriceps femoris	16.13	17.54	15.34	15.59	*		
Magnesium, mg/g [b]							
Liver	0.80	0.90	0.78	0.94		**	
Longissimus dorsi	0.89	0.94	0.95	0.98			
Psoas major	0.99	0.94	1.06	0.90			
Quadriceps femoris	0.97	1.02	0.99	0.88			
Calcium, mg/g [b]							
Liver	0.28	0.29	0.28	0.31			
Longissimus dorsi	0.16	0.15	0.18	0.17			
Psoas major	0.13	0.15	0.15	0.18			
Quadriceps femoris	0.17	0.14	0.18	0.15		*	
Non-protein nitrogen, mg/g [b]							
Liver	75.79	78.71	76.49	75.32			
Longissimus dorsi	12.49	13.40	14.38	15.05			
Psoas major	13.81	12.21	13.76	12.16			
Quadriceps femoris	14.89	14.24	14.71	14.63			

[a] Fat-free, carbohydrate-free basis.
[b] Fat-free, carbohydrate-free, dry matter basis.
[c] Fresh weight basis.
[d] Fat-free, dry matter basis.
*$p<.05$. **$p<.01$.

RESULTS AND DISCUSSION

Table 1 shows the effect of periodic electrical stimulation and limited feeding prior to slaughter on the chemical composition of the liver and certain beef muscles which included the longissimus dorsi, psoas major and quadriceps femoris. Limited feeding increased the moisture content of the liver and the three muscles studied while periodic electrical stimulation increased the moisture content of the liver and decreased the moisture content of the longissimus dorsi and quadriceps femoris muscles. Limited feeding and periodic electrical stimulation decreased the protein concentration of the liver. The concentration of ash in the liver was increased by limited feeding and by periodic electrical stimulation. Limited feeding increased the fat content of the liver. The above differences were very small and probably have no practical significance. The probable reason for this effect was the removal of the variation in fat, glycogen and lactic acid content of these tissues. The above changes in protein and ash concentration are difficult to explain because of the manner they were expressed. A change in one would automatically cause a change in the other.

The above data agree with Baur *et al.* (1959) who reported that the moisture content of the carcass was increased when pigs were deprived of calories only. This treatment also increased the fat content of the liver. However, Mitchell and Hamilton (1933) have reported that heavy exercise decreased the moisture content of the liver, heart and skeletal muscle of beef cattle.

Periodic electrical stimulation and limited feeding increased the glycogen content of the liver. There was an interaction between periodic electrical stimulation and limited feeding on the liver glycogen concentration because the combination of periodic electrical stimulation and limited feeding gave concentrations of liver that were larger than the additive effects of these two treatments alone. Limited feeding decreased the glycogen content of the longissimus dorsi and quadriceps femoris muscles while periodic electrical stimulation decreased the glycogen of all three muscles. Limited feeding and periodic electrical stimulation decreased the concentration of lactic acid in the psoas major and quadriceps femoris muscles. There was an interaction between periodic electrical stimulation and limited feeding on the lactic acid concentration of the quadriceps femoris muscle because the combination of periodic electrical stimulation and limited feeding gave concentrations of lactic acid in the quadriceps femoris muscle that were smaller than the additive effects of these two treatments alone. Lewis *et al.* (1961) showed that stress prior to slaughter decreased the glycogen concentration of the psoas major and the lactic acid concentration of the psoas major and quadriceps femoris muscles of hogs. These workers (Lewis *et al.*, 1962a) reported that stress prior to slaughter increased protein concentration in the psoas major muscle, decreased concentrations of lactic acid in the psoas major and quadriceps femoris muscles and the glycogen and sugar phosphorus concentrations in the psoas major muscles. Gibbons and Rose (1950) suggested that fatigued hogs have livers lower in glycogen and have psoas major muscles that contain less lactic acid than rested hogs. Sayre *et al.* (1961) reported that cold water stress decreases lactic acid concentration of the gluteus medius muscle in hogs. Rose and Peterson (1951) showed that exercise decreases lactic acid in rats. Mitchell and Hamilton (1933) reported that the effect of long continued muscle exercise decreased liver fat and the moisture in the liver, heart, and skeletal muscles. The probable reason for the increased glycogen concentration of the liver due to limited feeding and periodic electrical stimulation was that the animals converted the lactic acid produced in the muscle into glycogen in the liver. This conversion has been reported by Cori (1931). The decrease in protein concentration in the liver due to periodic electrical stimulation prior to slaughter would occur due to the action of the adrenal steroids in producing energy from liver protein (Turner, 1955).

Limited feeding increased the inorganic phosphorus of the liver and psoas major muscle while periodic electrical stimulation increased the inorganic phosphorus in all three muscles. This agrees with Hall *et al.*

(1944) who reported that dark-cutting beef had very little glycogen and large amounts of inorganic phosphate. Limited feeding decreased the sugar phosphorus in the quadriceps femoris muscle while periodic electrical stimulation decreased the sugar phosphorus in the longissimus dorsi and the quadriceps femoris muscles. Limited feeding increased the total phosphorus in the liver and decreased the total phosphorus in the quadriceps femoris muscle. Since the above increases in inorganic phosphorus were greater than the decreases in sugar phosphorus, this indicates that some of the inorganic phosphorus was coming from some source other than sugar phosphorus. Limited feeding and periodic electrical stimulation increased the potassium in the liver and limited feeding decreased the potassium content of the quadriceps femoris muscle. This decrease in muscle potassium agrees with Benjamin *et al.* (1961) who showed that the stress of anoxia, heat and cold resulted in a decrease of the potassium concentration in the brain of rats. Baur *et al.* (1959) reported that the deprivation of water and calories, calories only and water only caused increases in the sodium concentration and decreases in the potassium concentration of pig carcasses. Since there was no significant effect of these treatments on the sodium content it would seem that potassium shifts from the cells and interstitual fluid into the plasma to be eliminated in the urine while sodium is retained in an effort to maintain hemostasis (Turner, 1955). Since there were increases in the potassium content of the liver, it seemed that some of the potassium that left the muscles was accumulated in the liver.

Periodic electrical stimulation increased the magnesium content of the liver and decreased the calcium content of the quadriceps femoris muscle.

Periodic electrical stimulation increased the weight of the liver by 0.9 of a pound while limited feeding decreased the weight of the liver by one pound (Lewis *et al.*, 1962b). Since these changes were observed it seemed desirable to determine the effect of these treatments on the total amount of the various chemical components in the liver. This effect is shown in Table 2. Periodic electrical stimulation increased the total amount of moisture, ash, glycogen, potassium, and magnesium in the liver.

Limited feeding decreased all of the chemical components studied except fat and glycogen. The total amount of glycogen was increased by limited feeding and there were no effects of any of the treatments on the total amount of fat in the liver. As was true on the concentration basis, there was

Table 2. Effect of stress and limited feeding prior to slaughter on the absolute weights of various chemical components of liver (av. of six animals).

	Full ration		Limited ration		Significant variation due to:		
	Not stimulated	Stimulated	Not stimulated	Stimulated	Limited	Stress	Interaction
Moisture, lb	6.0	6.2	5.1	5.7	**	**	
Protein, lb	1.8	1.8	1.4	1.4	**		
Ash, lb	0.121	0.122	0.095	0.104	**	*	
Fat, lb	0.09	0.17	0.20	0.26			
Glycogen, g	24	42	50	124	**	**	**
Lactic acid, g	6.8	7.0	5.4	6.1	**		
Inorganic phosphorus, g	3.4	3.6	3.0	3.0	**		
Sugar phosphorus, g	2.0	2.1	1.4	1.6	**		
Total phosphorus, g	14	14	11	12	**		
Sodium, g	2.2	2.1	1.5	1.7	**		
Potassium, g	12	13	10	12	**	*	
Magnesium, g	0.70	0.78	0.52	0.65	**	**	
Calcium, g	0.24	0.25	0.19	0.21	*		
Non-protein nitrogen, g	6.5	6.7	5.1	5.2	**		

* $p < .05$.
** $p < .01$.

an interaction between stress and limited feeding on the total amount of glycogen in the liver.

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Protein Solubility as Influenced by Physiological Conditions in the Muscle[a, b, c]

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SUMMARY

The solubilities of sarcoplasmic and myofibrillar proteins were determined at the time of slaughter, onset of rigor mortis, completion of rigor mortis and 24 hr after death in muscles exhibiting a wide range of physiological conditions during the post-mortem period. Muscle protein solubility was grossly altered by the conditions of both temperature and pH which existed at the onset of rigor mortis or during the first few hours after death. Sarcoplasmic protein solubility at 24 hr was decreased to 55% of that found at 0 hr in muscle groups exhibiting high temperature and low pH at the onset of rigor mortis. Conversely, only a 17% reduction of sarcoplasmic protein solubility was noted in groups with high pH at onset. Myofibrillar protein solubility ranged from no reduction during the first 24 hr after death when pH remained high at onset to 75% reduction in muscle with low pH and high temperature at the onset of rigor mortis. The 24-hr pH of the muscle appeared to have only a minor influence on protein solubility. Muscle protein solubility appeared to be one of the major factors affecting the juice-retaining properties of muscle.

INTRODUCTION

Several studies have been conducted to determine the effects of extraction methodology upon muscle protein solubility at the time of death (Helander, 1957; Hasselbach and Schneider, 1951). The solubility of sarcoplasmic and myofibrillar proteins has been used to characterize protein changes during animal maturity (Dickerson, 1960; Needham, 1931; Dickerson and Widdowson, 1960), and compare protein compositions of different muscles (Hill, 1962; Lawrie, 1961). However, the post-mortem changes in muscle protein solubility have received only limited study (Crepax, 1951; Partmann, 1963) since the early work of Mirsky (1936). Bendall and Wismer-Pedersen (1962) have suggested that rapid glycolysis, which resulted in pale, soft, exudative muscle, directly affected only the solubility of sarcoplasmic proteins. Those workers (Bendall and Wismer-Pedersen, 1962) postulated that sarcoplasmic protein denatured and precipitated on the fibrillar proteins and thereby reduced fibrillar protein solubility. The purposes of this experiment were to: 1) study the changes in sarcoplasmic and myofibrillar protein solubility during the first 24 hr post-mortem and relate these changes to the time course and conditions of rigor mortis and glycolysis; and 2) determine the extent to which protein solubility was associated with ultimate color, structure, and juice retention of the muscle.

EXPERIMENTAL

Longissimus dorsi muscles from 15 market-weight pigs were used in this experiment. The time course of rigor mortis was approximated on an excised strip of muscle with the use of a "rigorometer" described by Briskey *et al.* (1962). Onset of rigor mortis was defined as the time at which the muscle started to lose elasticity; rigor mortis was complete when the muscle had lost all of its

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[c] This investigation was partially conducted during residence of E. J. Briskey as F. C. Vibrans Senior Scientist Fellow at the American Meat Institute Foundation.

extensibility. Samples for protein extraction were frozen in a dry ice-acetone bath immediately after slaughter, at the onset of rigor mortis, at the completion of rigor mortis, and at 24 hr post-mortem (Briskey and Sayre, 1963). These samples were held at −30°C for not more than 72 hr, sectioned with a microtome at 20 μ, and maintained at −20°C until extraction at 2°C (Helander, 1957). Sarcoplasmic proteins were extracted with 0.03*M* K phosphate, pH 7.4; and total soluble proteins were extracted with 1.1*M* KI in 0.1*M* K phosphate, pH 7.4. Myofibrillar proteins were calculated as the difference between the amounts of total soluble proteins and sarcoplasmic proteins. Nonprotein nitrogen was measured after trichloroacetic acid precipitation. Surface reflectance, pH, and muscle temperature were measured at specified intervals post-mortem (Sayre *et al.*, 1963b). The juice-retaining capacity of muscle chilled 24 hr was measured by the filter-paper moisture-absorption technique of Grau and Hamm (1953) as modified by Urbin *et al.* (1962) and was expressed as the ratio of total area to meat-film area (Sayre *et al.*, 1963b).

RESULTS AND DISCUSSION

All samples were categorized according to the acidity and temperature of the longissimus dorsi at the onset of rigor mortis (Table 1). The muscles from two or three pigs were categorized in each group. The pH of muscles in groups A and B fell to 5.6 or below at onset; however, muscle temperature remained above 35°C in group A but it was below 35°C in group B. Muscles in groups C and D were at an intermediate pH between 5.7 and 5.9 at onset, with the temperature of C above 35°C and that of D below 35°C. Onset of rigor mortis occurred at relatively high pH values of 6.0 or above in groups E and F. The short delay phase of muscle in group E resulted in onset temperatures above 35°C, whereas muscle temperatures in group F fell below 35°C prior to onset of rigor mortis.

Table 1. Physiological conditions at onset of rigor mortis.

Group	Temperature	pH
A	>35°C	5.3–5.6 (Low)
B	<35°C	
C	>35°C	5.7–5.9 (Med.)
D	<35°C	
E	>35°C	6.0+ (High)
F	<35°C	

pH, color and structure. Fig. 1 illustrates the time course of pH change in the previously described groups. pH values are shown for samples taken immediately after exsanguination, at onset of rigor mortis, at completion of rigor mortis, and after a 24-hr chilling period. Group A (low-pH high-temperature) muscles evidenced extremely rapid pH declines and the lowest pH values during the first 2 hr post-mortem. These rapid pH declines were similar to the accelerated pH reductions previously described (Briskey and Wismer-Pedersen, 1961; Sayre *et al.*, 1963a). Nevertheless, the pH values in group A were not as low at onset and completion of rigor mortis and at 24 hr as in group B (low-pH low-temperature). Glycolytic rate as indicated by pH decline with time post-mortem was similar for groups B (low-pH low-temperature) and C (medium-pH high-temperature), but the pH at onset and 24 hr was considerably higher in group C. Although groups C and D had similar pH values at onset, group D had a markedly lower ultimate pH. Glycolysis was only limited in group E (high-pH high-temperature), which resulted in high pH values (>6.0) at onset of rigor and ultimate pH values similar to those found initially in the first four groups. pH declined slowly in group F (high-pH low-temperature), and the onset of rigor mortis occurred while the pH remained high; however, these muscles ultimately (24 hr) achieved a rather low pH. Since the time course of rigor mortis was followed on an excised strip and pH deter-

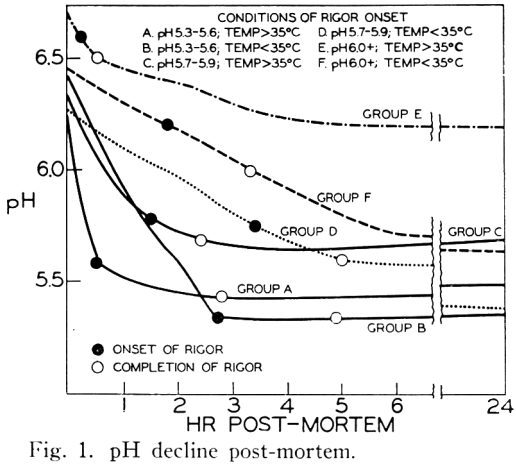

Fig. 1. pH decline post-mortem.

minations were made on adjacent areas in the intact muscle, the values are only approximations since wide differences have been noted in the composition of adjacent muscle areas (Lawrie, 1961).

Another indication (Fig. 2) of postmortem chemical change was the fading of the uniformly dark-red color found in all muscles at the time of death (Briskey *et al.*, 1959, 1960). Surface reflectance (485 mμ) was an objective measurement of color, with pale, soft, exudative muscles giving a high reflectance value.

Color fading (reflectance increase) was very rapid in group A (Fig. 2) and supports change in color and structure as previously noted by Briskey and Wismer-Pedersen (1961) under the conditions of rapid glycolysis. The rate and extent of color change was greater in group C than in B, indicating the importance of temperature as well as pH in altering the physical properties of muscle. This fact can be further illustrated by comparing groups C and D, which had the same pH but differed markedly in temperature at onset of rigor. As pH values at onset of rigor mortis remained above 6.0 (groups E and F), postmortem changes in surface reflectance were small and were in agreement with previously reported data (Briskey *et al.*, 1959; Sayre *et al.*, 1963a).

Protein solubility. Sarcoplasmic protein solubility (Fig. 3) decreased moderately to

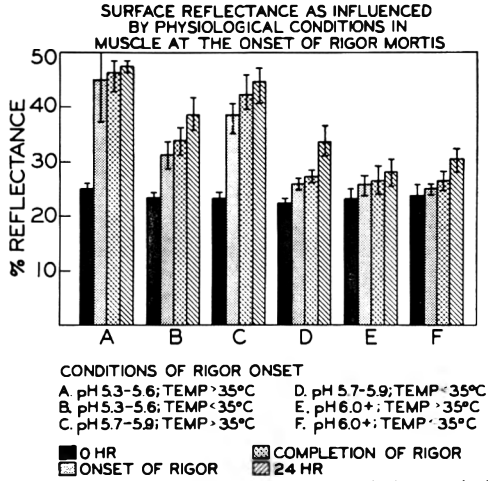

Fig. 2. Surface reflectance as influenced by physiological conditions in muscle at the onset of rigor mortis.

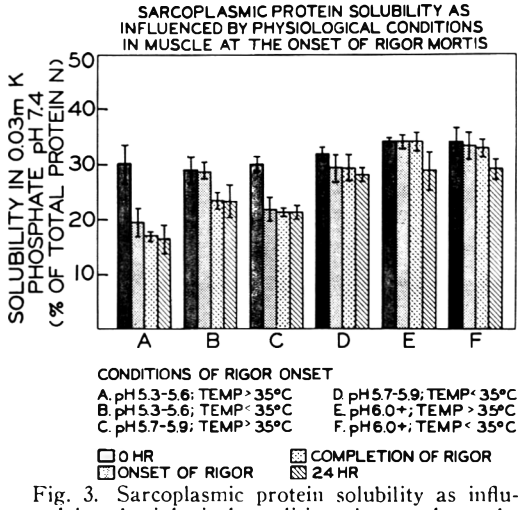

Fig. 3. Sarcoplasmic protein solubility as influenced by physiological conditions in muscle at the onset of rigor mortis.

extensively during the first 24 hr postmortem, regardless of physiological conditions at rigor onset. Extractable sarcoplasmic proteins in group A decreased at 24 hr to 55% of their original value. This loss in sarcoplasmic protein solubility occurred when acidity developed at high temperatures, as described by Bendall and Wismer-Pedersen (1962). The reduction in sarcoplasmic protein solubility was readily apparent at the onset of rigor mortis in both the low- and medium-pH groups as long as the temperature remained high (groups A and C), although the loss in solubility was not as marked as that reported by Bendall and Wismer-Pedersen (1962). It should be noted, however, that extraction procedures in the present experiment were rigorous whereas extraction procedures of Bendall and Wismer-Pedersen (1962) were limited. The difference between the magnitude of loss in sarcoplasmic protein solubility in these two experiments implies that sarcoplasmic proteins may become denatured in varying degrees, whereby it becomes more extractable under rigorous procedures. The solubility of sarcoplasmic proteins in groups D, E, and F was only slightly lower at 24 hr than the solubility values found initially in the first three groups.

Conversely, myofibrillar protein (Fig. 4) showed no loss in solubility under conditions of slow pH decline, regardless of temperature at the onset, as seen in groups E

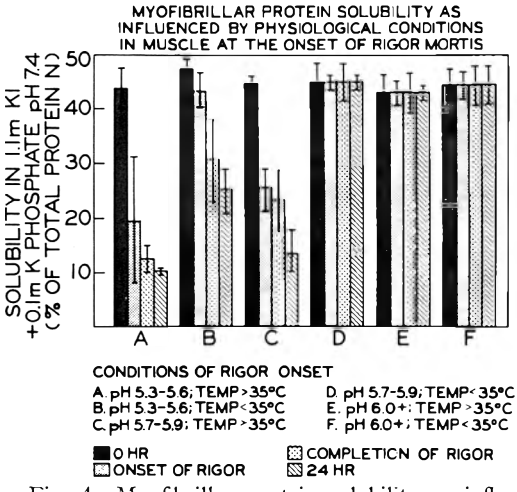

Fig. 4. Myofibrillar protein solubility as influenced by physiological conditions in muscle at the onset of rigor mortis.

and F. Likewise, there was no loss in myofibrillar solubility with a medium pH at onset as long as the temperature was low (group D). This observation is particularly interesting in view of the fact that the pH of both B and D groups fell to 5.3 at 24 hr even though myofibrillar solubility remained high in group D. However, under conditions of high temperature and a medium or low pH (groups A and C), loss of myofibrillar protein solubility was severe, as previously noted for watery pork (Bendall and Wismer-Pedersen, 1962). Less than 50% of the fibrillar protein was extractable in group A at the onset of rigor mortis, and only 25% was extractable after 24 hr. A major portion of decrease in total solubility was therefore attributable to the loss of solubility in the myofibrillar fraction, which is in agreement with the recent report of Connell (1962). Again, it is of interest to note that muscle pH values, both at onset and at 24 hr, were higher in groups A and C than in group B. Thus, high muscle temperature under medium- to low-pH conditions during the onset of rigor mortis and/or the first few hours after slaughter drastically reduced both sarcoplasmic and myofibrillar protein solubility. Data from these trials do not show whether the loss in myofibrillar protein solubility resulted from precipitation of sarcoplasmic proteins as described by Bendall and Wismer-Pedersen (1962) or from a direct alteration of the myofibrillar proteins. However, it should be pointed out that when rigor mortis occurred between pH 5.7 and 5.9 at temperatures below 35°C (group D), the sarcoplasmic protein solubility decreased approximately 12% without loss of myofibrillar protein solubility.

Correlations. Correlation coefficients (Table 2) show significant relationships between sarcoplasmic protein solubility and pH at onset ($p<0.01$) and completion ($p<0.05$) of rigor mortis. pH values at slaughter and at 24 hr were not associated significantly with sarcoplasmic protein solubility, again pointing out the importance of physiological conditions in the muscle as rigor mortis developed. Muscle temperature at death was closely associated with sarcoplasmic protein solubility; however, this association was not found with temperature at subsequent stages of rigor mortis. The fact that temperature at death was associated with sarcoplasmic protein solubility would appear to be an important observation, since Sayre *et al.* (1963a) have shown that pig muscle temperature can be easily elevated by ante-mortem treatment

Table 2. Correlations between protein solubility and physiological conditions in the muscle.

Correlations between:	pH				Temperature			
	0 hr[a]	Onset[b]	Completion[b]	24 hr[a]	0 hr	Onset	Completion	24 hr
Sarcoplasmic protein solubility (24 hr)[a]	.34	.64**	.55*	.33	−.78**	−.42	−.35	....
Myofibrillar protein solubility	.24	.52*	.44	.12	−.85**	−.64**	−.48	....
	Sarcoplasmic protein solubility				Myofibrillar protein solubility			
	0 hr	Onset	Completion	24 hr	0 hr	Onset	Completion	24 hr
Expressible Juice Ratio (24 hr)	−.53*	−.70**	−.75**	−.71**	.34	−.56	−.71**	−.62*

[a] Time post-mortem.
[b] Phase of rigor mortis.
* Indicates significance at the 5% level.
** Indicates significance at the 1% level.

immediately prior to death. Myofibrillar protein solubility (Table 2) was not associated as closely with pH as with muscle temperature. A highly significant ($p<0.01$) correlation was found between fibrillar protein solubility and initial temperature as well as muscle temperature at the onset of rigor mortis. Briskey and Sayre (1963) also noted that pH and temperature during the first two hr post-mortem were very important in determining protein solubility.

Correlation coefficients between the juice-retaining properties of chilled muscle (24 hr) and protein solubility during the early post-mortem periods (Table 2) indicate that muscle that ultimately became exudative began to lose sarcoplasmic protein solubility immediately after death. Conversely, fibrillar protein solubility was not affected as rapidly by the physiological conditions in the muscle after death. The relation between protein solubility and juice retention is in partial support of the observations on 24-hr muscle by Wismer-Pedersen (1959) and Hamm (1962).

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Electron Microscopy of Post-Mortem Changes in Porcine Muscle

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SUMMARY

Electron microscopy was used to follow changes in porcine muscle during the 24-hr post-mortem chilling period. Ante-mortem subjection to elevated temperatures or to elevated temperatures and then chilling was used to produce different rates and magnitudes of change in post-mortem muscle color, texture, and water binding. Normal muscle exhibited a gradual disruption of sarcoplasmic components, with little if any change in the myofibrils. Muscle that went into rigor rapidly at a low pH and high temperature ultimately appeared soft, pale, and watery, and electron micrographs revealed a rapid disruption of sarcoplasmic components and some disorganization of the myofilaments. Muscle that went into rigor rapidly at a high pH and a reduced temperature ultimately appeared dark, firm, and dry, and electron micrographs revealed a high degree of organization and preservation of myofibrillar structure.

INTRODUCTION

Post-mortem glycolysis proceeds at extremely variable rates in porcine tissue (Lawrie, 1960; Briskey and Wismer-Pedersen, 1961; Sayre *et al.*, 1963b). When the glycolytic rate is slow or when rigor mortis occurs at a high pH and/or a low temperature, the muscles appear dark and firm. When, however, glycolysis is accelerated and rigor mortis occurs at a low pH and high temperature, the muscle appears extremely pale, soft, and exudative (Briskey and Wismer-Pedersen, 1961; Bendall and Wismer-Pedersen, 1962; Briskey *et al.*, 1962) and the protein solubility is decreased markedly (Sayre and Briskey, 1963). The elucidation of muscle structure with the electron microscope has been thoroughly explored (Huxley, 1958) but little attention has been given to electron microscopic changes occurring in muscle when these glycolytic changes are taking place during the first 24 hr post-mortem. Most electron microscope work has been designed to obtain samples in the highest state of preservation possible. However, Moore *et al.* (1956) studied degeneration in muscle following tourniquet, and noticed some dissolution of the I bands and also of the Z lines.

This study was made to investigate post-mortem structural changes in porcine muscles that showed a severe loss of fluid following a 24-hr post-mortem chilling period. Because the rate of pH drop was known to influence 24-hr post-mortem appearance of porcine muscle, various ante-mortem treatments were employed to evoke different rates of post-mortem glycolysis.

MATERIALS AND METHODS

Samples of longissimus dorsi muscle obtained from swine of the Poland China breed were fixed in buffered osmic acid, dehydrated, embedded in a 4:1 mixture of prepolymerized butyl and methyl methacrylates, sectioned with glass knives on the Porter-Blum ultramicrotome, mounted on formvar film, and examined with a Siemens electron microscope. All pictures were taken at an original magnification of ×8,000. The animals were slaughtered in the normal manner, and within 45 min of death they were placed in a cooler at 1–4°C for chilling.

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[b] This investigation was supported in part by a research grant EF-81 (C3) from the Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, and by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation.

[c] Departments of Meat and Animal Science (Paper No. 379) and Biochemistry, cooperating.

Samples were removed from the same area of the longissimus dorsi simultaneous with stunning, at the onset of rigor mortis, and 24 hr post-mortem. pH values were determined with a single-probe glass electrode placed directly on the freshly cut muscle surface.

The phases of rigor mortis were measured with the "rigorometer" of Briskey *et al.* (1962). The delay phase was defined as the time from death of the animal until the first signs of loss of extensibility were observed. The onset phase was from the end of the delay phase until no more loss of extensibility was observed. Completion of rigor mortis was the combined time of the delay and onset phases.

Some animals were subjected to elevated temperatures immediately preslaughter by placing them in a controlled high-temperature chamber (Sayre *et al.*, 1963b) at 42–45°C for approximately 1 hr immediately ante-mortem. Other animals were subjected to the elevated temperatures and then placed directly in an ice-water bath (Kastenschmidt *et al.*, 1963) for approximately ½ hr prior to slaughter.

RESULTS

Electron microscopic observation of normal-appearing muscle showed a gradual disruption of sarcoplasmic components during the first 24 hr post-mortem. No marked alteration of the myofibrils was noticed. Fig. 1 shows the appearance of normal-appearing porcine muscle 24 hr post-mortem. A disruption of mitochondria is shown, but no obvious damage to the myofibrils from post-mortem glycolysis is noted. Normal porcine muscle undergoes a gradual pH drop to approximately 5.5 during the 24 hr post-mortem chilling period.

Figs. 2 and 3 respectively illustrate the appearance of muscle at the onset of rigor mortis and 24 hr post-mortem. These samples were taken from a pig that had been subjected to an elevated temperature and whose muscle temperature was 115°F at the time of death. The onset of rigor mortis occurred 40 min after death, and the muscle pH had already fallen to 5.6. By 24 hr post-mortem the muscle pH was 5.5, the visual color was quite pale, and the muscle was exuding large quantities of liquid.

Fig. 2 shows that most of the sarcoplasmic material had already been disrupted 40 min after the death of the animal. Although the A bands seem to be intact in the 24-hr sample (Fig. 2), there seems to have been some disruption in the I bands. It appears as if some of the I band filaments are clumped together.

Figs. 4, 5, and 6 respectively illustrate the appearance of muscle at death, at the onset of rigor, and 24 hr post-mortem from a heated pig whose muscle temperature reached 110°F. The onset of rigor mortis occurred at a pH of 5.6 *only* 15 min following death of the animal. Fig. 4 shows that mitochondria and other membranous

Fig. 1. Appearance of normal porcine muscle 24 hr post-mortem.

Fig. 2. Appearance of muscle at the onset of rigor mortis, from a pig previously subjected to an elevated temperature.

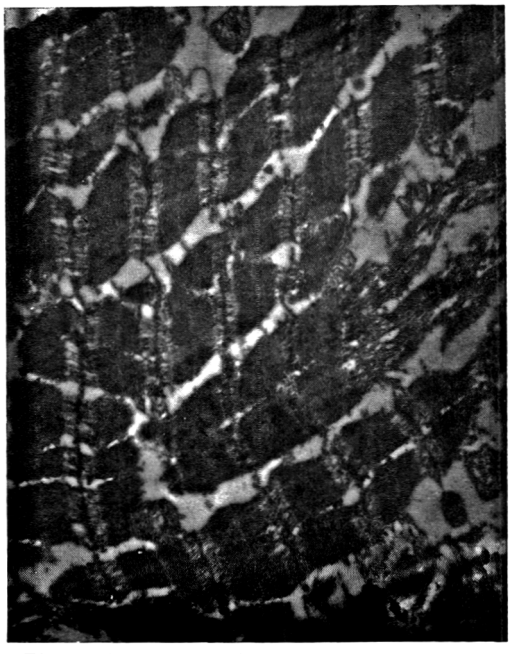

Fig. 3. Appearance of muscle 24 hr post-mortem from a pig previously subjected to an elevated temperature.

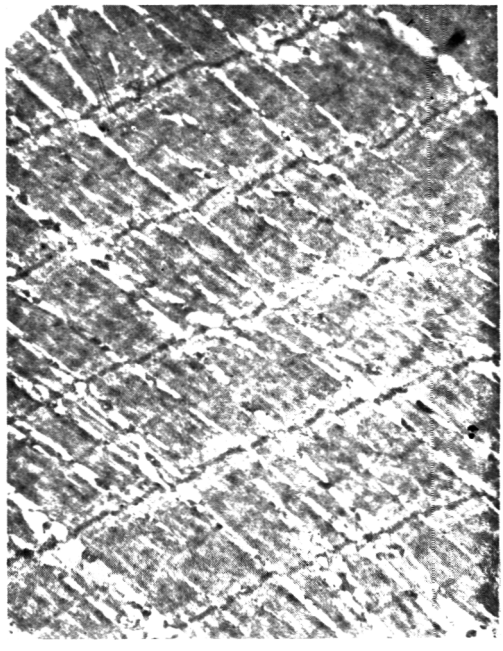

Fig. 5. Appearance of muscle at the onset of rigor mortis from a pig previously subjected to an elevated temperature.

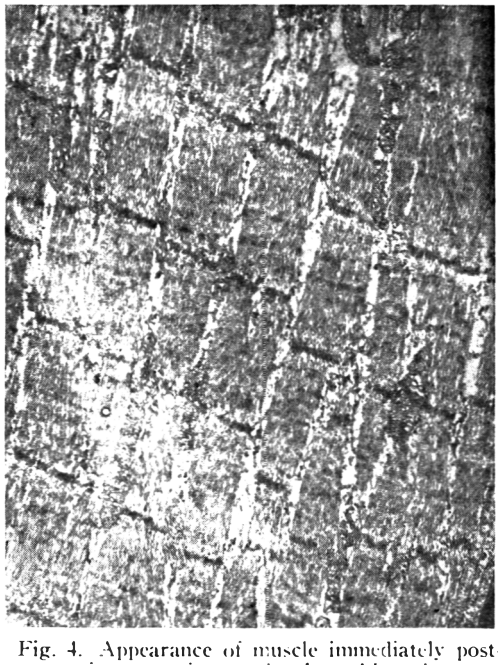

Fig. 4. Appearance of muscle immediately post-mortem from a pig previously subjected to an elevated temperature.

Fig. 6. Appearance of muscle 24 hr post-mortem from a pig previously subjected to an elevated temperature.

structures were present in the sarcoplasm. The muscle appears to be highly contracted, as evidenced by the short I bands. Only 15 min later (Fig. 5) the sarcoplasm had been almost completely disrupted. After 24 hr the muscle visually appeared extremely pale, the fiber bundles seemed to be disconnected, and the muscle was losing excessive amounts of fluid. The electron micro-

graph at this time (Fig. 6) shows a granular appearance of the A band. This would indicate a disruption of the protein filaments, and the logical conclusion would be to relate the loss of water binding to a breakdown of the muscle proteins. The more distinct band pattern in Fig. 6 may be the result of extraction of soluble substances during post-mortem anaerobic glycolysis and aging.

Fig. 7 illustrates a 24-hr post-mortem sample from an animal that had been heated and then chilled immediately preslaughter. The muscle temperature at death was 96°F. and the onset of rigor mortis occurred only 20 min after death, but at a high pH of 6.6. The 24-hr pH was 5.9, the muscle visually appeared slightly darker than normal, and the cut surface of the meat was free from exudative fluid. The electron micrograph (Fig. 7) showed an especially high retention of myofibrillar organization and structure.

Fig. 7. Appearance of muscle 24 hr post-mortem from a pig previously subjected to an elevated temperature and then to chilling in a cold bath.

DISCUSSION

Changes in porcine muscle structure during the first 24-hr post-mortem include a disruption of sarcoplasmic components and some disorganization in the myofibrils. The myofibrillar disorganization occurred in muscle from porcine animals that had been subjected to elevated temperatures immediately preslaughter. Briskey and Wismer-Pedersen (1961) explained the significance of rate of pH fall and muscle temperature in the ultimate appearance of porcine muscle, and, more recently, Briskey *et al.* (1962) discussed the importance of onset of rigor mortis at different levels of pH to the ultimate appearance of muscle.

The present study substantiated that a rapid onset of rigor at a low pH and high temperature is predisposing to a pale, soft watery muscle at 24-hr post-mortem. Moreover, electron micrographs revealed that, under these conditions, disruption of sarcoplasmic components is extremely rapid, and in one case an obvious disruption of the muscle myofilaments was evident. This indicates that the basic problem of post-mortem muscle properties and appearance lies in the changes in the muscle proteins caused by a rapid onset of rigor mortis at a low pH and high temperature.

Bendall and Wismer-Pedersen (1962) have shown irregular bands (light microscope) in muscles which have undergone an extremely rapid rate of glycolysis. Those workers postulated that the irregular bands represented sarcoplasmic proteins which had been denatured by acidity and temperature. Cassens *et al.* (1963a,b) have more recently observed irregular bands at the time of death in muscles which were ultimately normal. These bands also appeared similar in micro and ultra structure to the contracture bands of thaw rigor. Additionally, Cassens *et al.* (1963 a,b) studied these bands extensively with a phase and electron microscope and concluded that the irregular bands were primarily myofibrillar in nature. When the irregular bands occurred in muscle with normal glycolysis they appeared to be reversible; however, Forrest *et al.* (1963) noted in one sample that when the irregular bands appeared pre-rigor in muscle with rapid glycolysis they were also evident in the 24-hr post-mortem sample.

Conversely, if the muscle went into rigor mortis rapidly and at a high pH, dark firm muscle resulted, which, examined with the electron microscope 24 hr post-mortem, appeared to be in a high state of preservation. The effect of lowering the muscle temperature immediately prior to slaughter was thought to influence the post-mortem appear-

ance of muscle either through slowing glycolysis or through enhanced preservation by the actual lower muscle temperature before death. The combined effect of ante-mortem heating and then chilling on the processes occurring post-mortem in muscle is not fully understood.

Post-mortem muscle appearance may be affected by ante-mortem treatment, and the subsequent post-mortem changes sustained by the muscle may, in part, be detected with the electron microscope.

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RESEARCH NOTE

Some Properties of Acetoin-2,4-dinitrophenylhydrazone

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Lindsay *et al.* (1962) recently reported new physical properties of acetoin-2,4-dinitrophenylhydrazone (2,4-DNP) (see Table 1). Not only are these data different from our results but the ultraviolet adsorption values are unusual for a 2,4-DNP (see, e.g., Jones *et al.*, 1956). To prevent confusion from entering the literature concerning this 2,4-DNP, frequently isolated from natural products, we have attempted to elucidate the structure of Lindsay's "new derivative."

We first established that our acetoin-2,4-DNP remained unchanged after following Lindsay's chromatographic procedures. This excluded any hypothesis of transformation of acetoin-2,4-DNP during this experimental step. Our attention was then drawn to the fact that, in the hands of Lindsay *et al.*, acetoin yielded a mixture of diacetyl-bis- and diacetyl-mono-2,4-DNP beside their new "acetoin-2,4-DNP," "regardless of the reaction conditions." These by-products may originate from the well known oxidation of the originally formed acetoin-2,4-DNP by an excess of 2,4-dinitrophenylhydrazine. The latter at the same time is reduced to ammonia and 2,4-dinitroaniline.

As can be seen in Table 1, the properties reported by Lindsay *et al.* (1962) for their "acetoin-2,4-DNP" are identical with those of 2,4-dinitroaniline. The color reaction with alcoholic KOH is also bright pink as reported by the authors, whereas authentic acetoin- and acetol-2,4-DNP give a characteristic intense brown color under these conditions. The infrared spectrum doublet at 3333 and 3460 cm^{-1} cannot, as the authors state, be attributed to the OH vibration. These two sharp bands are characteristic for the NH stretching vibration of primary amines (Cross, 1960). We might point out that infrared spectra of 2,4-DNPs of acetoin (KBr pellets) (Winter *et al.*, 1963) and also of acetol show no characteristic OH vibration in the 3500 cm^{-1} region. In solution, only a very weak band is observed. In the 1080 cm^{-1} region, however, a strong to medium-strong vibration does appear that seems to be characteristic for α-hydroxy-2,4-DNPs (see Table 1).

Our conclusion is that the properties of acetoin-2,4-DNPs are those mentioned by Day *et al.* (1960) and us (see Table 1), and that this derivative is easily distinguishable from diacetyl-mono-2,4-DNP. We

Table 1. Some properties of the substances discussed.

Substance	Melting point (°C)	λmax chloroform [a]	λmax alcoholic KOH [a]	Characteristic infrared absorption bands (KBr pellets) 3300–3500 cm^{-1} region	Characteristic infrared absorption bands (KBr pellets) 1070 cm^{-1} region
"Acetoin-2,4-DNP" Lindsey *et al.*	178–179	327, 259	510, 382	3333, 3460	?
2,4-Dinitroaniline [b]	181	327, 259	514, 382	3340, 3460	none
Authentic acetoin-2,4-DNP	114 [c]	357 [c]			
	108–109	357, 243	433, 234	3310	1072
(−)-Acetoin-2,4-DNP [d]	114.5–116	357, 243	432, 236	3320	1070
Acetol-2,4-DNP	134.5–136	355, 244	433, 235	3320	1082
Diacetyl-mono-2,4-DNP	175–176	353, 245	504, 234	3290	none

[a] Jones *et al.*, 1956.
[b] Commercial sample, purified by crystallization.
[c] Day *et al.*, 1960.
[d] Winter *et al.*, 1963.

should like to add that an acetoin-2,4-DNP free of diacetyl-mono- and diacetyl-bis-2,4-DNP is obtained by using a slight excess of acetoin with respect to the reagent.

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Modification of Radiolethality by Vitamin K_5 and Certain Analogs in Model Systems and in Foods[a]

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SUMMARY

Spores of *Bacillus subtilis* var. *niger* and *B. stearothermophilus* irradiated in nitrogen were killed in greater numbers in the presence of vitamin K_5, 4-amino-1-naphthol, or 2-amino-1-naphthol than when irradiated without chemical. When irradiation was performed in air, the chemicals were without effect, or even protective. Spores of both organisms were particularly sensitive when irradiated in nitrogen with 4-amino-1-naphthol.

Irradiation of *Micrococcus radiodurans* in anoxia with these naphthol derivatives gave losses in cell recovery that resulted in much lower populations than could be attributed to chemical toxicity *per se*. These chemicals were found to be toxic to a yeast that had been isolated from frozen orange juice when the preparations were in buffer of pH 7.0 and 4.0. When the yeast was suspended in orange juice, on the other hand, the chemicals were neither toxic nor radiosensitizers. Milk was also found to interfere with the bactericidal and radiolethal activities of these chemicals. Sulfhydryl compounds were shown to decrease radiolethal action.

INTRODUCTION

A number of compounds have been shown to increase radiolethality. Among these are *N*-ethylmaleimide (Bridges, 1961), iodoacetic acid, and phenylmercuric acetate (Bridges, 1962). These three have a strong affinity for combining with sulfhydryl groups, considered to be of great importance in radiation repair mechanisms. In two investigations, El-Tabey Shehata (1961) and Silverman *et al.* (1962) have shown that another class of compounds, related to and including vitamin K_5, are also capable of increasing radiolethality. In common with Bridges' findings for *N*-ethylmaleimide, their effectiveness in general was more pronounced under anoxia.

It was thought desirable to study the ability of K_5 and two structurally related analogs to modify the radiosurvival of radiation-resistant microbial species in addition to microbial populations in milk and orange juice.

EXPERIMENTAL

Organisms. Spores of *Bacillus subtilis* var. *niger* and *B. stearothermophilus* were grown, harvested, and assayed by procedures previously described (Davis *et al.*, 1963). Preparation of washed cells of *Streptococcus faecalis* 10C1 (ATCC 11700) and *Micrococcus radiodurans* (obtained from A. W. Anderson) were as in previous studies (El-Tabey Shehata, 1961) except that 0.18*M* instead of 0.66*M* phosphate buffer, pH 7.0, was used. The yeast was isolated from orange juice after plating an orange serum agar (Difco). Washed and standardized cell suspensions of the isolate were obtained from 48-hr shake cultures grown in plate count broth supplemented with 5% orange juice. The yeast cells were washed twice with 0.18*M* buffer (pH 7.0) and standardized by optical density to give a final yeast population of about 6×10^7 cells per ml. The viable cell count was determined on orange serum agar after 48 hr of incubation at 30°C. The total plate counts for both raw and pasteurized milks containing *S. faecalis* organisms were determined in plate count agar, the incubation period being 3 days at 30°C.

Chemicals. Chemicals were obtained from the sources indicated: Vitamin K_5 (2-methyl-4-amino-1-naphthol, Parke, Davis and Co.), 4-amino-1-naphthol and 1-amino-2-naphthol (Eastman Organic Chemicals), l-cysteine (Calbiochem), and glutathione (Schwarz Bioresearch, Inc.).

[a] Contribution no. 531 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology.

Irradiation. When the organisms were irradiated in ampoules, the techniques of El-Tabey Shehata (1961) and Silverman *et al.* (1962) were used. Unlike filtered orange juice or buffer, milk formed excessive foam when sparged. To alleviate this problem, capped vials were substituted for glass ampoules, antifoam (antifoam 66, General Electric) was added, and the air or nitrogen used in sparging was introduced through hypodermic needles. The irradiation source was of the submerged type, the gamma rays originating from cobalt-60 rods emitting an average of 5500 rad per min.

RESULTS AND DISCUSSION

Radiation-resistant organisms (Microbial spores and Micrococcus radiodurans). Table 1 shows the survival fraction of spores of *B. subtilis* var. *niger* and *B. stearothermophilus* irradiated in a nitrogen atmosphere in solutions of Vitamin K_5, 4-amino-1-naphthol (4A1N), or 2-amino-1-naphthol (2A1N). In the presence of air, a protective effect by the added substances was noted. The radiolethal effect did not seem to be concentration-dependent, and 150 ppm was not, in general, three times as effective as 50 ppm. None of the three chemicals were toxic to *B. subtilis* var. *niger*, but they were, to varying extents, toxic to *B. stearothermophilus*. In agreement with previous studies with vegetative cells (El-Tabey Shehata, 1961; Silverman *et al.*, 1962), the magnitude of this toxicity was greater in air than in nitrogen. The radiolethal activity, on the other hand, was most evident under conditions of anoxia.

Bridges (1961) exposed *Escherichia coli*, *Staphylococcus aureus*, a *Pseudomonas* sp., and spores of *Bacillus subtilis* to *N*-ethylmaleimide (NEM), and found that NEM was an effective radiosensitizer against *E. coli* and *S. aureus* in either air or nitrogen and only under anoxia for the *Pseudomonas* sp. Contrary to the present findings, Bridges found NEM ineffective for decreasing radiosurvival of *B. subtilis* spores.

Vitamin K_5 (Reynolds and Lichtenstein, 1950; Verona, 1955) and vitamin K_3 (Shimidu and Ueno, 1956) were reported to decrease the thermal resistance of spores. Michener *et al.* (1959) found that although vitamins K_1 and K_5 failed to reduce the heat

Table 1. The influence of Vitamin K_5, 4-amino-1-naphthol, and 1-amino-2-naphthol on the radiosurvival of spores of *Bacillus subtilis* var. *niger* and *Bacillus stearothermophilus*.

		Irradiated; survival fraction [a]								*B. stearothermophilus*	
		Bacillus subtilis var. *niger*				*Bacillus stearothermophilus*				Unirradiated	
		Air		Nitrogen		Air		Nitrogen		Survival	
Chemical	ppm	(134,000 rad)	(256,000 rad)	(256,000 rad)	(488,000 rad)	(305,000 rad)	(488,000 rad)	(488,000 rad)	(671,000 rad)	Air (%)	Nitrogen (%)
Vitamin K_5	0	0.09	0.010	0.10	0.010	0.14	0.012	0.06	0.006	100	100
	50	0.14	0.020	0.05	0.003	0.17	0.010	0.019	0.002	43	47
	150	0.13	0.030	0.05	0.0009	0.12	0.15	0.03	0.002	33	26
4-amino-1-naphthol	0	0.11	0.011	0.11	0.011	0.05	0.005	0.10	0.010	100	100
	50	0.10	0.030	0.05	0.003	0.20	0.030	0.06	0.003	31	61
	150	0.13	0.020	0.03	0.0013	0.20	0.020	0.025	0.002	39	83
1-amino-2-naphthol	0	0.11	0.011	0.12	0.014	0.10	0.008	0.01	0.018	100	100
	50	0.15	0.020	0.07	0.007	0.19	0.022	0.027	0.009	48	59
	150	0.17	0.030	0.07	0.003	0.16	0.021	0.032	0.008	59	72

[a] Survival fraction = number of viable microorganisms after irradiation/original cell population.

resistance of spores at 200 to 500 ppm, vitamin K_4 (2-methyl-1,4-naphthalenediol diacetate) did. The mechanisms involved in thermal and radiation destruction of microorganisms have not been elucidated. Physiological similarities have been noted between the destruction of spores by heat and irradiation (Levinson and Hyatt, 1960), but this should not be confused with comparative differences in susceptibility among microbial species to heat or irradiation. Morgan and Reed (1954) and Schmidt *et al.* (1962) showed that spores of *Clostridium botulinum*, by being extremely radioresistant, would be a main concern in irradiated foods. This organism is not the most heat-resistant species in thermal processing. *B. subtilis* var. *niger* was less radioresistant than *B. stearothermophilus*. If both organisms were irradiated to yield comparable survival fractions, then 50 ppm of the naphthalene compounds caused an additional and essentially equal decrease in survival in both cases.

M. radiodurans was much more radioresistant than *B. subtilis* var. *niger* or *B. stearothermophilus* (Table 2). It is nevertheless not notably heat resistant (Niven, 1958), and in this study shows extreme susceptibility to the toxic effects of 4A1N and 1A2N in air and to 4A1N in nitrogen. In fact, as regards toxicity, *M. radiodurans* is one of the more susceptible vegetative organisms examined. Nevertheless, a reduction of at least 3 log cycles was noted when these chemicals were present during radiation under anoxia.

Yeast. An unidentified yeast originally isolated from orange juice was examined in buffer and in freshly reconstituted frozen orange juice. It appears to be less resistant in buffer than El-Tabey Shehata (1961) reported for *Torulopsis rosea* (Table 3). This work substantiated his conclusions for *T. rosea* in that this class of compound appeared to be more effective against yeast when irradiation was conducted in air rather than in nitrogen. Both K_5 and 4A1N in buffer were toxic to the yeast isolated; in fact 4A1N was too toxic to be evaluated. In citrate buffer, pH 4.0, K_5 and 4A1N proved to be too toxic for

Table 2. Effect of irradiation of suspensions of *Micrococcus radiodurans* in solutions of Vitamin K_5 and certain analogs.

		Irradiated		Unirradiated	
		Survival fraction		Survival	
Chemical	ppm	Air (483,000 rad)	Nitrogen (720,000 rad)	Air (%)	Nitrogen (%)
Vitamin K_5	0	0.52	0.46	100	100
	150	0.035	0.0013	77	92
4-amino-1-naphthol	0	.....	0.32		
	10	.....	0.0013	T [a]	28
1-amino-2-naphthol	0	.....	0.30		100
	30	.....	0.00031	T	92

[a] T = too toxic for evaluation.

Table 3. Neutralization of the radiolethal effects of Vitamin K_5 and 4-amino-1-naphthol (4A1N) on a yeast suspended in orange juice.

		Irradiated		Unirradiated	
		Survival fraction		Survival	
	Chemical (40 ppm)	Air (28,000 rad)	Nitrogen (84,000 rad)	Air (%)	Nitrogen (%)
Buffer (pH 7.0)	None	0.15	0.054	100	100
	K_5	0.00041	0.0074	38	60
	4A1N	.....	.....	T [a]	T
Orange juice (pH 3.7)	None	0.13	0.62	100	100
	K_5	0.31	0.52	78	100
	4A1N	0.25	0.79	84	92

[a] T = too toxic for evaluation.

Table 4. A comparison of the ability of naphthol derivatives to reduce, during irradiation, the natural microbial populations in raw milk and of *Streptococcus faecalis* added to pasteurized milk.

Chemical	Survival fraction: Raw milk [a] Air (25,000 rad)	Raw milk [a] Nitrogen (68,000 rad)	*S. faecalis* in pasteurized milk [b] Air (25,000 rad)	*S. faecalis* in pasteurized milk [b] Nitrogen (75,000 rad)
None	0.30	0.11	0.40	0.10
K_5	0.18	0.035	0.46	0.053
4A1N	0.15	0.022	0.30	0.0026
1A2N	0.18	0.076	0.40	0.0019

[a] The raw milk contained 62,000 organisms per ml.
[b] Approximately 450,000 *S. faecalis* cells per ml were added to milk containing 560 organisms per ml.

use in these experiments. Vitamin K_5 has been recommended for the preservation of fruit juices (Yang *et al.*, 1962). In this study, there were no bactericidal effects by K_5 or 4A1N against the yeast cells in orange juice, nor any modification of radiosurvival due to K_5 or 4A1N.

Milk. A typical experiment with raw milk is shown in Table 4. None of the three compounds displayed any toxicity toward the natural flora in raw milk, and 4A1N caused the largest radiolethal effect under anoxia. Interestingly, 4A1N and 1A2N, but not K_5, were more effective as radiosensitizers against *S. faecalis* organisms added to pasteurized milk than to the natural flora of raw milk. This decrease occurred only in nitrogen, not in the presence of air, and no bactericidal effect was evident. Wilkowske *et al.* (1955) had noted that although 3 ppm of vitamin K will decrease acid production by dairy starters, as much as 100 ppm was necessary to arrest acid development for 24 hr at 30°C.

There appears to be some constituent(s) present in both milk and orange juice capable of ameliorating or eliminating the toxic effects and radiation effectiveness of these compounds. El-Tabey Shehata (1961) and Silverman *et al.* (1962) suggested that proteins or sulfhydryl groups are among the more obvious compounds capable of reacting or combining with K_5, 4A1N, or 1A2N and therefore capable of minimizing their influence.

Model systems. To determine the influence of sulfhydryl groups and certain proteins, a number of model systems were examined (Table 5). Both cysteine and glutathione, which are protective compounds, eliminated an appreciable portion of the radiolethal activity of K_5 and 4A1N in nitrogen. The activity of 4A1N was decreased more than that of K_5 by these sulfhydryl compounds. A sulfhydryl-equivalent amount of the tripeptide glutathione was not notably more efficient than cysteine, the main activity residing in the sulfhydryl moiety. Casein and gelatin did not alter the activity of K_5 or 4A1N (Table 6) appreciably, but the addition of cysteine decreased the activity of these naphthalene compounds. Casein itself is somewhat protective to *S. faecalis*, since the survival fraction (Table 6) was 0.39 with casein and 0.29 in buffer (Table 5). Gelatin did not protect the cells when it was used alone (Table 6) since the magnitude of the survival fraction was

Table 5. Modification of the radiolethal activity of Vitamin K_5 and 4-amino-1-naphthol (4A1N) by cysteine and glutathione on *Streptococcus faecalis* irradiated in nitrogen.

Chemical (ppm) Glutathione	Cysteine	K_5	4A1N	Irradiated survival fraction (88,000 rad)
	0	0		0.29
	14	0		0.38
	28	0		0.45
	14	20		0.00022
	28	20		0.0023
	0	20		0.00075
	0		20	0.000015
	28		20	0.013
98		0	0	0.49
98		20	0	0.0083
98		0	20	0.013

Table 6. The survival of *Streptococcus faecalis* cells suspended in casein or gelatin solutions containing combinations of K_5, 4-amino-1-naphthol (4A1N), and cysteine, after irradiation in a nitrogen atmosphere.

Chemical	Survival fraction (88,000 rad)	
	Casein (3%)	Gelatin (0.1%)
None	0.39	0.26
K_5 (40 ppm)	0.0026	0.0018
4A1N (40 ppm)	0.0014	0.0011
Cysteine (28 ppm)	0.72	0.39
K_5 + cysteine	0.068	0.014
4A1N + cysteine	0.32	0.028

comparable with gelatin and buffer. It is therefore likely that beta-lactoglobulin in milk, the primary source of free sulfhydryl groups (Jenness and Patton, 1959), is primarily responsible for the inactivation of K_5, 4A1N, or 1A2N in milk.

This study points up the difficulty of attempting to find a radiosensitizing compound universally applicable to all foods. This is due to complexity of the flora usually found in foods, and to the diverse nature of the food constituents. Nevertheless, this attempt, although illustrating certain of the shortcomings inherent to radiosensitization of microorganisms, should not discourage further investigations. Increasing our knowledge of the exact mechanisms involved may, it is hoped, eventually be applicable to amplifying the biological effects of radiation and result in decreasing the irradiation dose required to minimize microbial populations in foods.

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Staphylococci in Food and Food Intoxication
A Review and an Appraisal of Phage Typing Results

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SUMMARY

A review has been made of published information on the phage groups and types of staphylococci concerned in food-borne intoxication. It revealed that the majority belonged to phage group III with the types 6 and 47, either alone or with others, being the most common. In Great Britain the yearly incidence of food-borne intoxication, from 1950 to 1962, due to strains of phage group III ranged from 64.5 to 94.7%.

In food handlers incriminated in outbreaks the nose was the most common focus of infection, and the hand came next.

Meat and milk were the foods most commonly incriminated. Group III phages lysed 64.3% of strains found in meat and 58.1% of those in milk. Group IV phage lysed nine times as many milk-borne as meat-borne strains, which were also quite common in milk taken direct from cows, with and without mastitis or abnormal secretion.

The meager data on staphylococcal contamination of "wholesome" food, meat, milk and fish, were tabulated. A high proportion of these strains were untypable, but one-fourth to one-third of the strains were lysed by phages of Group III.

The results of phage typing of staphylococci from milk, dairy workers, market milk and dairy products were summarized. Phages obtained from strains of human and animal origin have been used for typing purposes. Of the phages in the "international set" obtained from staphylococci of human origin, 42D, Group IV, lysed more strains than any other phage. A set of phages of bovine origin has not yet been internationally recognized.

About two-thirds of the strains obtained from bovine mastitis but three-fourths of strains from normal milk were lysed by the typing phages employed. Group III phages lysed two-and-one-half times as many strains from normal as from mastitis secretion.

Cheese, butter, butter-milk, skim milk, cream, ice cream, kefir, and dried and condensed milk have yielded staphylococci lysed mainly by phages of Groups III and IV.

Dairy workers, veterinary surgeons, and farmers have yielded strains similarly lysed. Some of the people in these groups as well as the animals they tended have suffered clinical disease processes, due to strains with identical phage reactions.

Phage-typable strains have been found in the noses of cattle, in dogs, pigs and chickens; their human attendants have, in some cases, carried identical strains.

The proportion of staphylococci, lysed by the 52/52A/80/81 complex of phages has shown a twofold increase in hospitalized human patients and a two-and-one-half increase in animals in the last few years. Staphylococci lysed by this complex are regarded as particularly invasive in and pathogenic for man.

No comparison has appeared on the frequency distribution of staphylococci among the phage groups from food-borne intoxication and food handlers. A comparison was therefore made of such reported strains from: i) food causing intoxication; ii) the nose; iii) the feces; and iv) superficial lesions of apparently healthy persons. The main features were: a) the low proportion of strains lysed by phages of Group III in ii, iii, and iv; b) the low proportion of untypable strains, especially in i.

Systematic phage typing of staphylococci from wholesome food, food incriminated in intoxication outbreaks, hospital patients and the general population in defined geographical areas is recommended. Sites such as the nose, hands, feces, and perineum of healthy people and disease processes in hospitalized patients should be searched for staphylococci, which then should be typed by the use of a standard technique with the aid of the international set of phages from strains of human origin, augmented, where necessary, by phages from strains of animal origin.

In addition to the international set of "human" phages, it would be useful to establish and use an international set of phages of animal, especially bovine, origin.

Phage typing cannot be used to determine whether a given staphylococcus has produced or can be induced to produce enterotoxin; but is an excellent means of assisting in determining whether staphylococci from victims of food-borne intoxication, suspected food and suspected food handlers are related.

INTRODUCTION

Two methods may be used in the study of the circumstances leading to staphylococcal intoxication of man: i) To obtain maximum information on all important factors of "epidemiological cases" as they occur; and ii) To determine what factors in the common foods, their preparation, handling, storage, and consumption may lead to contamination with enterotoxin-producing staphylococci.

The factors in *i* have been studied to some degree, but those in *ii* are largely neglected, yet, both methods are equally important.

It is generally accepted that only coagulase-positive staphylococci producing enterotoxin cause food-borne intoxication. But their presence cannot he readily and unequivocally demonstrated. Indeed, "progress in research of staphylococcal food-poisoning is stifled almost to extinction by the difficulty of testing for the toxin"—to quote van Heyningen (1950).

Usually its presence only becomes apparent when intoxication has already occurred, and then the causal organism may be no longer extant.

Whilst there is no definite and easy method of demonstrating the toxin or the strains producing it, at least one useful method exists of determining whether strains isolated from the incriminated food, the victims, and the original source are related, if not identical. Phage typing is such a method.

The fragmentary nature of the available data makes the task of review somewhat difficult. There are several reasons for this: In cases of reported intoxication the search for strains in the various kinds of food that have been consumed may yield few isolates, or the phage typing may suggest that several strains were involved. The personnel concerned with the preparation of the incriminated food may not yield any strains or the strains found may no longer be of the same phage type; or the sites in man searched for staphylococci may not be the right ones, especially when clinical signs of infection are absent. Or the number of samples of vomitus or feces from the victims may be insufficient.

Nearly two decades have elapsed since phage typing was first applied to staphylococci. Although it has been used successfully for strains suspected of causing food intoxication (for summary, see Munch-Petersen, 1960), the method is still not widely used, except in Great Britain, perhaps because it entails the necessity of maintaining "typing phages" (which may mutate), or perhaps its uses and limitations are still not well known.

Yet, it is rather more than a useful adjunct to the biochemical and physiological tests normally applied to staphylococci from all the habitats or sources involved in food-borne intoxication. Indeed, phage typing

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has been described as a sensitive epidemiological tool (Blair, 1956).

This paper is intended to survey published information, to make an appraisal of phage typing of staphylococci in food and food intoxication of man, and to compare the frequency distribution of the phage groups represented by staphylococci incriminated in outbreaks of food-borne intoxications and in other habitats.

HISTORICAL REVIEW

A brief review of the main features of the development of phage typing will be given; excellent and exhaustive reviews and appraisals have appeared (Williams and Rippon, 1952; Anderson and Williams, 1956; Rippon, 1956; Brandis, 1957; Elek, 1959; Parker, 1962).

A peculiar degenerative change was observed in staphylococci isolated from vaccine lymph (Twort, 1915). The cause of this effect, which became known as bacteriophage lysis, was readily and indefinitely transmitted.

Use was made of this phenomenon to assist in tracing relationship among strains obtained from different sites (Burnet and Lush, 1935; Williams and Timmins, 1938).

A search was made for phages caried by staphylococci obtained from different sources, and attempts were made to systematize the findings (Fisk, 1942a, b; Fisk and Mordvin, 1944).

Details were prepared for the notation, systematic propagation and uses of phages and their reactions (Wilson and Atkinson, 1945; Williams and Rippon, 1952). Modifications in techniques and notations created to meet local requirements or to widen the scope of typing appeared (Wallmark, 1949; 1954; Wahl and Lapeyre-Mensignac, 1950a, b; Wahl and Fouace, 1952, 1954a, b; Blair and Carr, 1953; Hood, 1953; Vogelsang, 1953; Jackson *et al.*, 1954; Goldberg, 1954; Oswald and Reedy, 1954; Pöhn, 1955; Tarr, 1958; Lidwel, 1959).

Most staphylococci carry phages (Fisk, 1942a, b), some even as many as five (Rountree, 1949a), and most coagulase-positive strains are lysogenic, i.e., capable of being lysed (Gorrill and Gray, 1956), but are themselves immune to the phages they carry. Individual staphylococci can be made resistant to different phages in artificial cultures and some may change their apparent type (Smith, 1948a, b).

Phages were isolated from suitable staphylococci and designated by a number, or adapted from suitable phages and a letter was added to the designation. The set of phages used at any one typing center has changed over the years (e.g., Williams and Jevons, 1961).

Phages may be grouped serologically and some of the groups are closely associated with the animal origin of the strains (Rountree, 1949b; Rippon, 1956).

Phage filtrates are used in typing, usually in "routine typing dilutions" (RTD), where necessary augmented by 100 or 1,000 × RTD (Williams and Rippon, 1952; Wahl and Fouace, 1952, 1954a, b).

Routine test dilution is defined as the highest dilution at which the phage gives confluent lysis on the propagating strain and is the dilution used for routine phage typing.

Undiluted phage filtrates may induce virulent mutants of another prophage (Williams and Rippon, 1952; Rountree, 1956), or may contain agents inhibitory to phage growth thus leading to false reactions (Williams and Rippon, 1952; Rosenblum and Jackson, 1957).

Strains may be temporarily or permanently fully or partially resistant to phages which might otherwise lyse them. This partial resistance was shown by 154 untypable routine strains with undiluted filtrates of routine typing phages; 76 (49.4%) were lysed by one or more of the filtrates (Williams and Rippon, 1952).

The typable proportion of one collection of coagulase-positive staphylococci depends, *inter alia*, upon: i) the method, ii) the phages, and iii) the dilution of the phage filtrates, as well as on the strains. Thus, with the aid of 18 phages, 60.4% of 460 cultures were divided into 21 types; a further 22.6% were attacked by the phages, but did not fit into the defined types, and 17% were untypable (Wilson and Atkinson, 1945). Of 567 strains from various sources (including food-borne intoxication), 335 (59.1%) were typable, but 180 (31.4%) gave no reaction at RTD and 52 gave non-specific lysis (Williams and Rippon, 1952).

The isolation of fresh phages active against previously untypable strains may reduce their proportion. Thus, 30 out of 455 strains selected at random were untypable with the original set of phages; with 13 new ones, untypable strains were reduced to seven (Wahl and Fouace, 1954c).

Strains originating in man and untypable by the set of phages commonly used may become typable when phages from bovine strains are used (e.g., Hausler, *et al.*, 1960).

Some strains are untypable because they are resistant to phages. Since these are more common in the normal nose (39.8% of 265) or skin (24.5% of 302) (Williams and Rippon, 1952), this observation is particularly important in the search for the original human foci of staphylococci that have caused intoxication.

Staphylococci may be distinguished in two ways by the aid of phage-typing: 1) they may be "type-specific," that is lysed by only a single phage, or 2) they may be lysed by several phages, giving rise to a "pattern of lysis." An example may be quoted: Of 229 strains 118 (51.5%) were lysed *strongly* by a single phage, the remainder strongly by more than one phage. Phage pattern formation is not usually random; each phage is commonly associated with certain other phages, rarely with others. The frequency of occurrence of phage reaction patterns has been studied (Williams and Rippon, 1952).

At least 82 distinct patterns could be recognized among 229 strains, relying on strong lysis only, and 132 distinct patterns were observed among 567 strains typed with 24 different phages (Williams and Rippon, 1952).

On the basis of their susceptibility to various combinations of 16 phages, 103 strains were assigned to 30 different groups (Fisher, 1945).

With the aid of 20 phages 541 strains revealed 129 patterns. But two-thirds of a set of strains retested after an interval showed variation in phage patterns of plus or minus one strong reaction and in 10% of retests the same group was not obtained (Jackson *et al.*, 1954).

The technical steps now taken in phage typing technique may be outlined thus:

Phages are propagated on suitable liquid or solid media. Stock cultures and phages are used as starting material for the propagation of each new batch. To retain them unchanged they are freeze-dried. The phage titer is established by placing one drop of phage diluted in tenfold steps on the surface of an agar plate already spread with a 4–5-hour broth culture of the propagating strain and incubated at 30°C overnight. The RTD is then determined, i.e., the highest dilution that just fails to give confluent complete lysis. Sterilization is achieved by filtration through sintered glass or porcelain.

The lytic spectrum is determined by testing the phage against its propagating strain and a set of 16 standard test strains, so chosen that phages with similar lytic spectra can be differentiated.

Routine testing is done by inoculating the whole surface of an agar plate with the pure culture of the coagulase-positive staphylococcus under test, drying the plate, dropping phages at RTD on the plate in a predetermined grid, dry and read the confluent lysis after a suitable period. If no reaction is obtained the test is repeated with phage filtrates at higher concentration (Blair and Williams, 1961).

A number of the commonly used phages have been grouped together, because of the similarity of their lytic reaction. The various types in the currently accepted groups (together termed the International Set) are:—

Group I : 29, 52, 52A, 79, 80
Group II : 3A, 3B, 3C, 55, 71
Group III : 6, 7, 42E, 47, 53, 54, 75, 77
Group IV : 42D
Miscellaneous: 81, 187

Staphylococci not lysed by the usual phages are termed "Untypable." The above set is used for routine typing, but any laboratory may use additional phages found to be locally useful. In every case pure cultures of coagulase-positive strains must be used; phages are always used at RTD first, and further tests with more concentrated phages are made when no significant lysis is produced. At least 27 countries have now adopted this set of phages and an additional four countries have provisionally accepted the set (Blair and Williams, 1961, 1962).

A correlation has been sought between phage grouping and other characteristics of staphylococci:

i) The three major phage groups exhibited some correlation with Cowan's (1938) serological groups (Hobbs, 1948; Wahl and Fouace, 1952; Williams and Rippon, 1952). This relation was accepted only for Group III (Oeding and Vogelsang, 1954). Strains from food intoxication outbreaks showed less variations in both phage types and serotypes than did strains from hospital epidemics and other sources (Oeding and Williams, 1958).

ii) It has been noted (Parker, 1962) that staphylococci with certain phage patterns having a characteristic pathogenicity or epidemic virulence may possess certain other specific cultural characteristics, e.g., ability to cause opacity in egg yolk (Gillespie and Alder, 1952) or in horse serum (Tomlinson and Parker, 1956), to produce certain antibiotic substances (Parker *et al.*, 1955) or to resist the action of mercuric chloride (Moore, 1960; Green, 1962).

On the other hand, phage typing cannot be used to determine whether a given strain of staphylococcus produced or will produce enterotoxin (Anderson and Williams, 1956). Production of enterotoxin appears to be restricted to certain phage groups (Hobbs, 1948; Gentzkow, 1958), and in the main to phage groups III and IV (Allison, *et al.*, 1949; Williams *et al.*, 1953). Some intoxicating strains not typable by standard phages did yield a specific cat emetic, when a fractionation procedure was used; filtrates from the same cultures were emetic to monkeys (Thatcher and Robinson, 1962).

Frequency distribution among phage groups of epidemic staphylococci. *In general.* Table 1 is

Table 1. Frequency distribution among phage groups of staphylococci in food-borne epidemics, in general.

Total no. of epidemics	Percentage in phage group							
	I	II	III	IV	Mixed	Unclassified	Mixed and Unclassified	Untypable
666	1.5	1.0	77.0	4.6	1.2	7.0	3.3	4.4

Note: "Mixed": Cultures were lysed by phages of two or more groups.
"Unclassified": Cultures were lysed by phages not classified in groups I to IV.
"Untypable": Cultures were not lysed by the phages used for typing. A few of the original descriptions did not contain this category.

prepared from 46 recorded investigations on phage groupings of staphylococci concerned in 666 epidemics of food intoxication (Allison, 1943; Cooper, 1943; Cruickshank, 1943; Duncan, 1944; Murphy and Edward, 1944; Williams *et al.*, 1946; Oddy and Clegg, 1947; Ritchie *et al.*, 1947; Grant and McMurray, 1948; Hobbs and Thomas, 1948; Miller, 1948; Allison *et al.*, 1949; Hobbs and Freeman, 1949; Anon, 1950; Cockburn, 1950; Drysdale, 1950; Cockburn and Simpson, 1951, 1954; Hobbs, 1951, 1955; Hauge, 1951, 1952; St. Martin *et al.*, 1951; Parker, 1953; Crowe, 1954; Steede and Smith, 1954; Cockburn and Vernon, 1955a, b, 1956, 1957, 1958, 1959, 1960, 1961; Anderson and Williams, 1956; Parker and Lapage, 1957; Ortel, 1958; Dixon and Gardner, 1959; Nefed'eva, 1959; Nefed'eva *et al.*, 1961; Cust and Finch, 1961; Vernon, 1962).

The table shows that staphylococci lysed by phages of Group III are by far the most common in food intoxication, those lysed by the phages in Groups I, II, and Mixed are rare and of the same order of magnitude, viz., 1% to 1.5% of the total; those of Groups IV and Untypable amounted to between 4% and 5%, and "Unclassified" to 7%.

Strains lysed by phages of Group IV [42D are sometimes called "bovine" strains (see later)]. In six of the outbreaks listed, staphylococci did appear to originate in the udder of cows and to be transmitted in milk, cream, cheese, butter or ice cream. On the other hand, four other epidemics due to staphylococci with a similar reaction appeared to originate in or be communicated by meat or vegetables. For the remainder, no information was given.

In Great Britain. Over the last decade the Public Health Laboratory Service of the British Ministry of Health has published a valuable series of reports on the results of phage typing of the staphylococci isolated from food-borne intoxication (Anon, 1950; Cockburn, 1950; Cockburn and Simpson, 1951, 1954; Cockburn and Vernon, 1955a, b, 1956, 1957, 1958, 1959, 1960, 1961; Vernon, 1962). The results are tabulated since they are most useful as illustrations (Table 2). The yearly frequency of strains lysed by Group III phages ranged from a minimum of 64.5% of 31 outbreaks, in 1961, to a maximum of 94.7% of 57 outbreaks, in 1957, but averaged 82.5% for all 366 outbreaks. Strains lysed by members of other phage groups were far less common.

Since 1959, strains falling into Phage Group IV have caused at least nine outbreaks.

Table 2. Frequency distribution among phage groups of staphylococci in food-borne epidemics, in Great Britain.

Year	No. typed	Number in group						
		I	II	III	IV	Mixtures	Uncertain	Untypable
1950	46	..	..	39	..	..	3	4
1954	48	..	..	41	..	..	2	5
1955	43	1	..	39	..	..	3	..
1956	40	2	2	31	..	..	5	..
1957	57	1	2	54	..	..	..	..
1958	30	2	..	23	..	3	2	..
1959	34	..	..	29	5	..	..	..
1960	37	3	..	26	2	1	..	5
1961	31	2	1	20	2	..	..	6
Total	366	11	5	302	9	4	15	20
%		3.0	1.3	82.5	2.4	1.1	4.1	5.5

The few numbers under the headings "Uncertain" and "Untypable" should be noted.

Phage groups and types represented in incriminated food. Two vehicles for epidemic staphylococci were more common than any others, namely meat (especially in Great Britain) and milk (especially in the United States of America). The following information was collated from published reports (Allison, 1943; Cooper, 1943; Duncan, 1944; Murphy and Edward, 1944; McClure and Miller, 1946; Macdonald, 1946; Oddy and Clegg, 1947; Ritchie *et al.*, 1947; Hobbs and Thomas, 1948; Miller, 1948; Hobbs and Freeman, 1949; Anon, 1950; Drysdale, 1950; Parker, 1953; Steede and Smith, 1954; Anderson and Stone, 1955; Cockburn and Vernon, 1955b, 1957, 1958, 1959, 1960, 1961; Dixon and Gardner, 1959; Thorne and Wallmark, 1960; Cust and Finch, 1961; Gullotti and Spanò, 1961; Steede and Iredale, 1962; Vernon, 1962). Table 3 shows the frequency distribution of the phage groups.

Strains found in milk were not lysed by phages in Groups I or II, but those lysed by Group IV phages were nine times as frequent in milk as in meat. Group III phages lysed 64.3% of the strains found in meat and 58.1% of those in milk. Strains lysed by phages in more than one group (Mixed) were more than three times as frequent in meat as in milk.

The most common phage types in Group I were: 29, 52, 79; in Group II: 3C and 71; in Group III, whether meat or milk was the vehicle: 6, 47, or 53; in Group IV, 42D for both vehicles; in "Mixed Group," 80, 6, and 7 for both vehicles, but in addition, 47 for meat, and 42D, 29, and 52 for milk. It should be noted that staphylococci of the types commonly regarded as highly invasive in and pathogenic for human beings, viz. 52, 52A and 80, were represented in both vehicles (see later).

In summary, then, the staphylococci predominating in food-intoxication outbreaks were of phage type 6, sometimes alone, but mostly combined with 47, or with other types when meat was the vehicle, but of type 42D when milk was the vehicle.

Foci of phage-typed epidemic staphylococci in food handlers. A number of reports contained details of the focus of phage-typed staphylococci in food handlers (Cooper, 1943; Cruickshank, 1943; Duncan, 1944; McClure and Miller, 1946; Macdonald, 1946; Williams *et al.*, 1946; Oddy and Clegg, 1947; Gillespie, 1947; Hobbs and Thomas, 1948; Hobbs and Freeman, 1949; Monaci, 1953; Parker, 1953; Crowe, 1954; Steede and Smith, 1954; Cockburn and Vernon, 1955b, 1956, 1958, 1961; Gullotti and Spanò, 1961; Nefed'eva *et al.*, 1961; personal observations, 1961–62). The details were tabulated (Table 4).

Taking all findings together, the table reveals that the nose of food handlers was one or the only original focus in a total of 73, and the hand in 47 epidemics.

Mention should be made of the fact that in man staphylococci can persist, and may even multiply, in the deeper layers of the skin, after they have disappeared from the body surface (Roodyn, 1960).

It would be an advantage if all sites mentioned in the table as well as the perineum and possibly feces, which may be a source of infection (Fairbrother and Southall, 1950), of suspected food handlers were also examined for the causal strain in epidemics.

Other workers mentioned the probable or even proved focus of infection in one or more food handlers responsible for epidemics, but gave no details (Belam, 1947; Grant and McMurray, 1948; Allison *et al.*, 1949; Anon, 1950; Cockburn and Simpson, 1951, 1954; Hauge, 1952; Williams *et al.*, 1953; Fee, 1961).

Illustrations of the uses of phage typing. Two food handlers in a bakehouse appeared to be incriminated in an outbreak. Staphylococci were ob-

Table 3. Frequency distribution of staphylococcal phage groups represented in meat or milk, vehicles in food intoxication.

	Number in phage group						
Vehicle	I	II	III	IV	Mixed	Untypable	Total
Meat	4	2	36	2	10	2	56
Milk	..	..	9	18	3	1	31

Table 4. Foci of the epidemic staphylococcus in food handlers.

	Nose only	Hand only	Cuts only	Skin only	Throat only	Mouth blisters only	Nose and hand	Nose and throat	Nose and other parts	Nose, hand, mouth
No. of cases	44	27	4	3	2	1	19	4	5	1

tained from both, viz., from the nose of A, who had a "light" infection, and from the nose, a stye, the ear, and hand of B, who had recently suffered from an ear abscess. Phage typing of all strains revealed that A, not B, yielded a strain identical with those found in the incriminated food and the victims (Ritchie *et al.*, 1947).

Dried milk was responsible for a severe outbreak. Bulked milk from farms yielded staphylococci (15,000 per ml) of phage type 42D/77/29/52. After preheating and evaporating the count was less than 500 per ml. But milk in the atomizer feed tank, maintained at 40°C, gave a count of 5,000,000 per ml of phage type 53/75+, which was also obtained from the victims (Hobbs, 1955). Hence, the atomizer tank, not the farm milk, was the source of the epidemic strain.

Swabs were taken from the hands of 40 dairy workers, and eight yielded staphylococci, five being of phage type 42D and three of other types. Phage type 42D was also found in milk from five of six cows and in 34 cases of bovine mastitis (Macdonald, 1946).

Staphylococci in cheese made from goat milk caused an epidemic. Phage typing revealed that the victims, the cheese, and the milk from the goat carried the same strain, which was different from that carried by the cheese maker (Macdonald, 1944).

A recent contribution (Parker, 1962) contained an excellent illustration of the use of phage-typing of epidemic strains, with a discussion on the limitations of this method.

Phage groups of staphylococci from wholesome food. Search for bacteria, particularly staphylococci, in wholesome food is rarely reported in the literature, and hence there are hardly any data on phage-typed staphylococci from the habitat. Doubtless the experience gained from work at the British Central Typing Laboratory is behind the categorical statement that "coagulase-positive staphylococci, particularly those of the food-poisoning type, are rarely found in normal foodstuffs" (Hobbs, 1951).

However, four reports dealt with staphylococci in meat (abbattoir and retail) and slaughtermen's tools, clothes, etc. (Ludlam, 1952; Jay, 1961, 1962; Ravenholt *et al.*, 1961), three with milk (Papavassiliou and Obiger, 1959; Reid and Wilson, 1959; Clark *et al.*, 1961), and one with fish (Silverman *et al.*, 1961). Table 5 shows the distribution of the staphylococci among the various phage groups.

The table reveals: i) a high proportion of untypable strains in all three groups of food, suggesting that they were not often of human origin; ii) a proportion of strains lysed by phages in Group I to be approximately 1:6:17 for strains from milk, meat, and fish, respectively; iii) strains of Group II of about the same order of magnitude in the three foods; iv) Group III to contain about the same proportion of strains from meat and milk, whereas those from fish amounted to about two-thirds of the figure for the other two foods; v) strains of Group IV appeared in proportion of about 1:2:13 for fish, meat, and milk.

In Canada (Thatcher, 1960) the phage patterns of numerically dominant staphylococci in milk and cheese (apparently wholesome) have changed since 1953, from 42D (Group IV) to patterns identical with those known to have caused human infections. Of a collection of staphylococci isolated from fatal or otherwise severe cases of postoperative gastroenteritis, 17 were identical in phage pattern with strains isolated from cheese.

The incidence of potentially pathogenic staphylococci in dairy products (apparently wholesome) at the consumer level was determined. Of the following four examples, the first comes from Scotland, the other three from U.S.A.

Strains lysed by various phages were observed in: i) 26 of 40 samples of "certified" milk, during a three-year survey in Glasgow; all were lysed by phage 42D (McCallum, 1960); ii) seven of 207 samples of various dairy or milk products; whipping cream, half-and-half cream and buttermilk yielded strains lysed by phage 81 (Foltz *et al.*, 1960); iii) 125 cheese samples, representing 20 different varieties, of which 95 yielded staphylococci. Nine strains from four varieties of cheese were coagulase-positive and seven were lysed by Group III phages (Mickelsen *et al.*, 1961); iv) at least one half of 134 strains from cheddar cheese in retail shops which had phage patterns involving Group III (Donnelly *et al.*, 1962).

Phage groups and types of staphylococci obtained from the dairying industry. The informa-

Table 5. Frequency distribution among phage groups of staphylococci from food not incriminated in epidemics.

Source	No. of strains	Percentage in group						
		I	II	III	IV	Mixed	Unclassified	Untypable
Meat	482	6.7	2.3	35.3	1.2	16.8		37.7
Milk	174	1.1	1.1	36.2	9.4	21.8	6.7	22.7
Fish	147	17.0	3.4	23.1	0.7	14.3		41.5

tion in the previous section needs amplification. Information about strains from the dairying industry is summarized, but unfortunately there appears to be none published from the meat industry, with one exception: Usually, the same phage types of *S. aureus* were found on the skin of pigs and on chickens as on their handlers, although details were not given (Smith and Crabb, 1960).

The staphylococci may originate in: i) dairy cattle, e.g., those suffering from mastitis or carrying the organisms on the skin of the udder, with or without clinical signs; or ii) man, e.g. farm workers and plant operatives in milk treatment plants or dairy products factories.

Differences between human and animal strains were noted early (Smith, 1948a) and were further explored with the aid of phage typing and other tests: The phage grouping of strains from human (total 204) and animal (total 50) sources was compared. It revealed that 98.8% of 85 strains of phage Group I were of human origin, compared with 1.2% of those of animal origin; for 26 strains in Group II the respective figures were 100% and 0; for 68 strains in Group III, 75% and 25%; for 12 unclassifiable strains, 66.7% and 33.3%; for 38 partially typable strains, 68.4% and 31.6%; and for 25 completely untypable strains, 36.0% and 64.0% (Levy *et al.*, 1953). Thus, with the phages available, a greater proportion of human than of animal strains could be grouped.

Phage typing of staphylococci from all the above-mentioned dairy sources has been reported (*Slanetz and Jawetz, 1941; Macdonald, 1946; *Smith, 1948a, b; Hauge, 1951, 1952; McLean, 1951; Price *et al.*, 1954; Asso, 1956; Barnum and Fuller, 1956; Rountree *et al.*, 1956; Edwards and Rippon, 1957; *Nakagawa, 1958, 1960a, b, c; *Seto and Wilson, 1958; *Coles and Eisenstark, 1959a, b, c; Hoffmann, 1959; Loken, 1959; *Malik and Singh, 1959; 1960a, b; Mondini and Dovadola, 1959; Mora and Cavrini, 1959; Papavassiliou and Obiger, 1959; Reid and Wilson, 1959; Hausler *et al.*, 1960; Mondini and Gasparini, 1960a, b; *Takagaki, 1960; Thörne and Wallmark, 1960; Clark *et al.*, 1961; *Davidson, 1961a, b, c; Fratta *et al.*, 1961; White and McDonald, 1961; McPhillips and Cunningham, 1962; St. George *et al.*, 1962; *Slanetz and Bartlett, 1962).

The majority used part or all of the international set of phages of human origin; those marked * used phages obtained from staphylococci of animal (mostly bovine) origin, either alone or with the "human" set.

As an example of the relative usefulness of typing with the human vis-à-vis a bovine ("Coles") set of phages the following may be quoted:

Of 21 cultures from Cheddar cheese, 17 belonged to 14 different patterns and four were untypable with the human set; 18 belonged to seven different patterns and three were untypable by the bovine phages. Of the four untypable by the human phages, three were typed by the bovine phages (Hausler *et al.*, 1960).

The proportion of strains lysed by phage group IV, type 42D, was 82% of 123 strains (Macdonald, 1946); and was 83% of 1,016 strains, but the total proportion typable was 93.3% (Smith, 1948a, b). Of 28 strains 71.4% were typable, and 20 belonged to 44A (McLean, 1951). Phage type 6/46 lysed a high proportion of bovine strains (Asso, 1956). With the phages available, the proportion typable was: 79.2% of 591 strains (Barnum and Fuller, 1956); 57.8% of 57 strains from one herd, but only 3.2% of 62 from another herd (Rountree *et al.*, 1956). Strains lysed by Group III, type 6/47, were present in milk from 43 mammary quarters of cows in one herd (Wallmark and Thörne, 1958); 23.0% of 514 strains were lysed by phage Group IV, but 26.5% by phages in Group III (Loken, 1959); 75% of an unstated total of bovine strains were lysed by phage Group IV (Hoffmann, 1959).

The combined results of eight reports on typing of strains from bovine mastitis with the aid of "human" phages were compared with those from: i) four reports on strains from "normal" milk; ii) one report (Mann, 1960) of strains from bovines, not specified (Table 6).

About two-thirds of the strains from bovine mastitis were lysed, compared to nearly three-quarters of those from normal milk. The propor-

Table 6. Frequency distribution among phage groups of staphylococci from bovine milk and mastitis secretion.

Source	No. of strains	Percentage of total in phage group						
		I	II	III	IV	Mixed	Unclassified	Untypable
Mastitis secretion	2,563	15.1	2.2	14.2	15.9	9.8	11.1	31.7
Normal milk	259	5.0	0.8	36.3	8.5	14.7	12.3	22.4
Unspecified, bovine	144	11.8		6.3	18.8	20.8	0.7	41.6

tion of strains in Group III (which contains the highest proportion of all food-intoxicating staphylococci) was about 2½ times as great for those from normal as from mastitis secretion. The results of Mann occupy an intermediate position, and the proportion of untyphable strains was high.

Thus the result of typing was variable, but the reasons are not clear. Possibly there may have been involved such factors as: i) relative inexperience with the precise details of typing procedure, ii) a relatively high proportion of old strains, iii) a high proportion of strains not susceptible to the phages used. In any case, it would be advantageous if this aspect could be further explored by properly planned experiment.

Comparative tests on strains from normal and abnormal secretion has apparently been done only twice; no material difference was found (Barnum and Fuller, 1956; Reid and Wilson, 1959).

Within any one herd of cattle there may be a complete relationship or no relationship between phage type, types of hemolysin produced and color of growth on blood agar, but variations in some of the characteristics of phosphatase-positive strains obtained from cases of bovine mastitis may occur (Sharpe *et al.*, 1962).

Type 42D, phage Group IV, lysed a higher proportion of strains than any other phage in any other group of the human set (Smith, 1948a; Macdonald, 1946; Price *et al.*, 1954; Hobbs, 1955; Rountree *et al.*, 1956, Thatcher and Simon, 1956; Edwards and Rippon, 1957; Coles and Eisenstark, 1959a, b, c; Hoffmann, 1959; Mondini and Dovadola, 1959; Thatcher *et al.*, 1959; Fleming and Paton, 1961), but type 44A, Miscellaneous Group, did so under other circumstances (McLean, 1951; Seto and Wilson, 1958; Reid and Wilson, 1959; St. George *et al.*, 1962), and type 47C, Group III, in yet other circumstances (Mondini and Gasparini, 1960a, b; personal observations, 1961–62).

Of dairy products incriminated in intoxication, cheese was the most common (Macdonald, 1944; Anon., 1950; Cockburn, 1950; Cockburn and Simpson, 1951, 1954; Parker, 1953; Cockburn and Vernon, 1955a, b, 1957, 1958, 1959, 1961; Hendricks *et al.*, 1959; Thatcher *et al.*, 1959; Allen and Stovall, 1960; Hausler *et al.*, 1960; Donnelly *et al.*, 1962; Vernon, 1962). Also mentioned were cream (Parker, 1953; Steede and Smith, 1954; Cockburn and Vernon, 1955b, 1960; Foltz *et al.*, 1960; Gullotti and Spanò, 1961), ice cream (Williams *et al.*, 1946; Anon., 1950; Cockburn, 1950; Cockburn and Simpson, 1951, 1954; Cockburn and Vernon, 1955a), butter and kefir (Nefed'eva *et al.*, 1961), butter milk (Foltz *et al.*, 1960), dried milk (Cockburn and Simpson, 1954; Cockburn and Vernon, 1955a, b; Hobbs, 1955) and condensed milk (Cockburn and Vernon, 1955a).

Strains from these vehicles were lysed by phage types similar to those lysing strains from milk, but those from spray-dried milk (Hobbs, 1955) and ice cream (Williams *et al.*, 1946) by phages usually lysing strains associated with diseases in man.

Dairy workers harbored staphylococci lysed by phages of Group III (Cockburn and Simpson, 1951; Thörne and Wallmark, 1960; personal observations, 1961–62), or Group IV (Macdonald, 1946; Williams *et al.*, 1946; Cockburn and Simpson, 1951, 1954; Anon., 1959). Also noted, however, have been strains lysed by phages of Group I (Macdonald, 1946; Zinn *et al.*, 1961), by phages of Group II (personal observations, 1961–61), or by phages belonging to two groups (Steede and Ireland, 1962). In a few cases the focus of infection was noted, and again the nose was the most frequent, followed by the hand.

Thus, disease in man and cattle due to carriage of identical strains of staphylococci, as evidenced by identical phage reactions, has been recorded. Closer scrutiny may possibly reveal a higher incidence of transmission of the staphylococcus from cow to man, or vice versa (although the latter may be a slow process, for which see the excellent illustration provided by Davidson, 1961c), and also a higher incidence of clinical or subclinical infections than has hitherto been accepted.

Frequency distribution in dairy products and clinical material. The distribution of phage groups in dairy products and clinical material was examined (Thatcher and Simon, 1956), and Table 7 shows the results.

Three items in this table are noteworthy: i) None of the "dairy" strains was lysed by phages in Groups I, II, III, or Mixed; ii) none of the "clinical" strains was lysed by the phage type of

Table 7. Frequency distribution among phage groups of staphylococci from human clinical material and dairy products.

Source	Total no. of strains	Percentage in group					
		I	II	III	IV	Mixed	Untypable
Clinical material	139	10.5	10.5	47.4	.....	31.6	21.0
Dairy products	124	.....	.....	.....	80.0	.....	20.0

From Thatcher & Simon (1956).

Group IV; and iii) the majority of the "dairy" strains were lysed by the phage of Group IV.

More work of this kind would be very useful, since it may well have a bearing on the severity of food intoxication outbreaks.

Attention should be drawn also to the fact that phage-typable staphylococci have been recorded in the noses of cattle (Rountree *et al.*, 1956; Pagano *et al.*, 1960; Morrison *et al.*, 1961). And since dogs may be present on premises where food is being prepared, it should be noted that they have also been found in the nose or on the skin of these animals (Rountree *et al.*, 1956; Mann, 1959; Rajulu *et al.*, 1960; Live and Nichols, 1961; Morrison *et al.*, 1961).

The public-health significance of staphylococcal infections in animals has also been considered (Courter and Galton, 1962).

Members of the veterinary profession may be particularly exposed to risks of staphylococcal infections due mainly to the 52/52A/80 complex, as witness epidemics in veterinary schools (Pagano *et al.*, 1960; Live and Nichols, 1961).

On the other hand, the majority of 109 staphylococci obtained from the nose of members of a rural community, in which dairy farming preponderated, were lysed by phage group II (20.2%), with 18.4% lysed by group III, 17.4% by group I, 3.7% by mixed groups I and III, and only 0.9% by group IV; 16.5% were unclassified, 13.7% not typable, but 9.2% were lysed by phage type 80/81. The data were obtained in September 1959 to February 1960 (Page *et al.*, 1962). A study was made of the phage-typed staphylococci harbored by rural hospital patients in lesions found at follow-up visits six months after discharge. The lesions then found were caused by the original phage type in 50% of the 15 patients, in 33% of 11 family contacts, and the patients' phage type was transferred to cattle in 8.3% (Smith *et al.*, 1963).

Staphylococci lysed by phage 80/81. Special mention is warranted of the apparent ascendency, during recent years, of staphylococci lysed by phage 80/81 (all of which seem to be particularly invasive, pathogenic to man, and resistant to most antibiotics).

Strains typing as 52/52A/80/81, 52/80/81, 52A/80/81, 80/81, 80, and 81 are closely related (Asheshov and Rippon, 1959; Rountree, 1959; Rountree and Asheshov, 1961). These phage types are here considered together, and are termed type 80.

In the annual series of reports on food intoxication from the British Ministry of Health, staphylococci lysed by 80/81 phages were mentioned, the vehicles being cheese and meat (Cockburn and Vernon, 1957, 1960; Vernon, 1962).

From the bovine udder were obtained similar strains: The proportion lysed was: 23.4% of 591 strains (Barnum and Fuller, 1956); 2.0% of 395 strains (Edwards and Rippon, 1957); 34% of 111 strains, all penicillin-resistant (Robertson *et al.*, 1957); 32.3% of 87 strains also lysed by phage 42D (Coles and Eisenstark, 1959a); 25.8% of 151 strains (Thatcher *et al.*, 1959); 32.8% of 144 strains (Mann, 1960), and 5.9% of 51 strains (Clark *et al.*, 1961).

From food-intoxication outbreaks in Great Britain during 1949 to 1959, 3.5% of 210 independent strains were lysed (Williams and Jevons, 1961).

Strains found in superficial skin lesions or the noses of dairy workers and their families, in milk of the cows they tended, in the milk cooler, the tank, etc., were also lysed by phage 80 (see Anon., 1959; Wallace *et al.*, 1960, 1962; Zinn *et al.*, 1961).

Summaries have been prepared of the progress of phage type 80 in man and animals. One report dealt with its progress in the lesions of hospital patients from 1954 to 1957 (Williams, 1959). Table 8 shows the results.

Thanks to the courtesy of Dr. M. T. Parker (Staphylococcal Reference Laboratory, Colindale, U.K.) its further progress as a proportion of the total number of the *seven most important* phage types found in lesions of hospital patients can be given (Table 9).

The two series of figures may not be strictly comparable. The figures in Table 8 were apparently compiled from material received from quite a wide geographical area of Great Britain, but those in Table 9 from a limited number of hospitals in which the presence of an epidemic was suspected. Also available (Table 10) was the progress of phage type 80 in animals from 1957 to 1959 (Pagano *et al.*, 1960).

The tables show that the proportion of strains lysed by the 52/52A/80/81 complex of phages increased two-fold in man and 2½-fold in animals in the last few years. The significance is not

Table 8. Progress of staphylococci of phage type 80 in lesions of hospital patients.

	1954	1955	1956	1957
No. of strains typed	987	586	748	1,482
Type 80 (%)	14.9	10.7	17.0	30.1

Table 9. Progress of staphylococci of phage type 80 in lesions of hospital patients suffering from infections with staphylococci of the seven most important phage types.

	1954–57	1958–59	1960	1961
No. of strains typed (seven most important types only)	227	329	393	370
Type 80 (%)	18.9	35.2	35.3	36.5

Table 10. Progress of staphylococci lysed by phage type 80 in animals.

	1957	1958	1959
Number of strains typed	183	174	45
Type 80 (%)	19	13	49

From Pagano *et al.*, 1960.

obvious, but the finding suggests that closer scrutiny of the staphylococcal strains causing clinical manifestations in man and animals may be needed.

Whereas there is little doubt that staphylococci lysed by this phage type have become very important in lesions of human beings, at least in hospitals, and possibly also in the general population, there is hardly any information regarding their importance in lesions of animals as compared to staphylococci lysed by other phage types.

In summary, then, it may be stated that information currently available does not permit accurate assessment of the relative or actual importance of staphylococci lysed by phage type 80 and cognate phages, although the importance would appear to be increasing. It would be very useful to obtain accurate data on this subject, for both man and animals, especially vis-à-vis food intoxication.

Frequency distribution in food intoxication, noses, and superficial lesions. No reference has been found that deals specifically with the relative importance of the nose and the hand of food handlers as sources of staphylococci vis-à-vis those from food incriminated in intoxication.

However, two studies were made on the carriage of coagulase-positive staphylococci in healthy food handlers, one in Italy (del Campo, 1957), and one in the United States of America (Millian *et al.*, 1960). Unfortunately neither study included phage typing of the strains.

The former was concerned with the incidence, pathological characteristics and antibiotic resistance of staphylococci obtained from the nose and feces of food handlers (whose examination is compulsory in Italy). A total of 524 males and 382 females between the ages of 17 and 67 years yielded (i) 220 nasal strains, of which 138 were coagulase-positive and (ii) 88 fecal strains, of which 55 were coagulase-positive. About one half of the strains in each group were antibiotic-resistant.

In the second study, (i) 147 white and (ii) 87 Negro females, and (iii) 271 white and (iv) 72 Negro males were randomly selected from applicants for food handling positions in institutions and public restaurants. Three different culture media were used and nasal swabs yielded coagulase-positive strains in 37% of *i*, 20% of *ii*, 38% of *iii*, and 10% of *iv*. Nasal carriage was significantly higher in white than in Negro people.

The duration of nasal carriage of the same phage type may be very long—even up to seven years (Roodyn, 1960).

A review has just appeared dealing with healthy carriage of *Staphylococcus aureus*, its prevalence and importance (Williams, 1963). It mentioned carriage on various skin sites which was the subject of three investigations, each involving 50 persons. The nose was the most common site and was involved in about 42%. Second in importance was the hand (40%) in one study, the chest (18%) in the second, and the perineum (22%) in the third study. Persistence of nasal carriage rate in 679 adults, as revealed in four separate studies, was as follows: The incidence ranged from 16% to 33% and averaged 25% persistent carriers, ranged from 6% to 38% and averaged about 20% in non-carriers; the remainder were intermittent or occasional carriers.

Additional information of the frequency distribution of staphylococci lysed by the various phage groups from the various sites was obtained by comparing the findings or organisms in: i) the noses of healthy persons or of outpatients at hospitals; ii) superficial lesions of the hands of similar segments of the general population; and iii) skin lesions of patients seen in general practice. For this purpose and merely to illustrate the relative importance of these sites, information from five reports was used:

A) Strains from: i) the noses of normal persons; ii) miscellaneous superficial lesions; iii)

food intoxication; and iv) feces (Williams *et al.*, 1953).

B) Strains from: i) the noses of patients on admission to hospital, children and impetigo contacts; ii) superficial and deep lesions in outpatients (Parker, 1958).

C) Strains from: i) the noses of healthy recruits; and ii) food-borne intoxications (Williams and Jevons, 1961).

D) Strains from: i) skin and subcutaneous tissues of patients in general practice; and ii) the noses of the same patients (Johnson *et al.*, 1960).

E) Strains from: i) the noses of healthy persons; ii) the noses of patients (here taken together); and iii) septic fingers (Wesley-James and Alder, 1961).

The reports designated A, B, C, and E originated in Great Britain, D in Australia. Table 11 gives the results.

The table shows: i) a reduction in the proportion of untypable strains from 1953 to 1961; ii) some variation in the relative frequency of the various phage groups—probably due, at least in part, to the greater diversity of strains now acknowledged as typable than was thought to be so a decade or so ago; and iii) the low proportion of strains in Group III, which generally contains the majority of food-intoxication strains. Strains from food-borne epidemics are again found mostly to be lysed by phages of Group III, with a smaller proportion untypable.

It would be very useful to compare strains from: i) normal human beings in the general population; ii) food handlers; and iii) food incriminated in epidemics—all from the same geographical locations.

Types in the nose vs. feces or superficial lesions. Hardly anything is known of the frequency distribution of staphylococci among the phage groups obtained from the nose and feces or superficial lesions on the skin of victims of food-borne epidemics. Resort must therefore be had to information obtained from other conditions, merely to provide useful illustrations.

Phage typing of staphylococci isolated simultaneously from the nose and from feces of 35 hospital patients revealed that both sites yielded the identical strain in 22 (nearly 63%). In five cases, strains from the two sites were untypable. Of 16 patients with strains of Group I in the nose, seven yielded the same type from the feces; of six yielding Group II from the nose, four did likewise from the feces; of 13 yielding Group III from the nose, nine did likewise from the feces (Matthias *et al.*, 1957).

Two reports are given of the occurrence of strains in the nose and lesions of the same persons. In Great Britain (Roodyn, 1954), 51% of 71 patients with boils, seen in general practice, had the same strain in both sites. In Australia, 23.5% of 1,614 patients carried the identical strain in both sites (Johnson *et al.*, 1960).

Neither the figures in Table 11 nor those above, as will be seen, are very helpful in determining which site in man, be he food handler or victim involved in a staphylococcal food-borne epidemic,

Table 11. Frequency distribution among phage groups of staphylococci from nose, superficial lesions, and food epidemics.

Reference	Year	Source	Total no. of strains	Percentage in phage group						
				I	II	III	IV	Misc.	Unclassified	Untypable
A	1953	Nose	252	21.8	13.1	17.5	.....	.....	5.6	42.0
B	1958	Nose	275	25.1	15.7	28.0	.....	.....	13.4	17.8
C	1961	Nose	710	29.0	24.8	14.9	0.3	13.1	6.1	11.8
D	1960	Nose	726	44.9[a]	13.6	15.4	0.6	0.7	7.3	17.5
E	1961	Nose	197	37.5	25.7	10.6	.....	13.2	.....	12.7
B	1958	Superficial lesions	169	26.6	27.2	22.5	.....	.....	5.9	17.7
D	1960	Superficial lesions	1,614	54.7[b]	14.9	14.4	0.5	0.8	5.3	9.4
E	1961	Superficial lesions	123	45.5	24.4	8.1	.....	13.0	.....	8.9
A[c]	1953	Food epidemics	81	1.2	1.2	82.8	.....	.....	1.2	13.6
C	1961	Food epidemics	210	3.5	2.4	73.8	3.3	11.9	1.0	4.3

[a] Phage Type 80/81 (35.0%) included.
[b] Phage Type 80/81 (46.6%) included.
[c] Consists of 40 strains from cases with same phage type in food and victims, and 41 strains from cases with phage type from food *or* victims, but not from both.

A, Williams *et al.*, 1953.
B, Parker, 1958.
C, Williams and Jevons, 1961.
D, Johnson *et al.*, 1960.
E, Wesley-James and Alder, 1961.

should preferably be searched for the organism. However, it is to be hoped that this will be the subject of investigation.

CONCLUSIONS

A contribution emanating from the British Staphylococcal Reference Centre contained recommendations for phage typing of staphylococci of food-borne intoxications (Anderson and Williams, 1956). These are restated and slightly amplified below:

i) Reproducibility of typing of staphylococci from victims, food, and food handlers in food-borne intoxication epidemics is better than for those from hospital epidemics.

ii) Difficulty in interpretation is rare.

iii) Since isolation of staphylococci from vomitus may be difficult, feces of victims should be examined. However, since some 15% of normal people may carry staphylococci in feces (Allison, 1943), food-poisoning staphylococcus lysed by the same phage types should be recovered from a sufficient number of, but at least two, victims before a connection with those found in food can be accepted.

iv) When such a connection has been established and the source of the food has been found, phage typing of such staphylococci as may be obtained from the food handlers will assist in determining their causal role.

v) Phage typing cannot be used to determine whether a given staphylococcus produces enterotoxin.

vi) Strains should be phage-typed without delay, since typing ability may be lost on storage (Borchardt, 1958).

Some food-borne strains causing intoxication not typable by standard phages, did yield a specific cat emetic enterotoxin, when a fractionation procedure was used to obtain the toxic fraction; and filtrates from the same cultures were emetic to monkeys (Thatcher and Robinson, 1962). However, it does not necessarily follow that man would have been similarly affected.

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The Effect of Heating Time and Temperature on the Shear of Beef Semitendinosus Muscle [a]

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SUMMARY

The effect of time and temperature on the shear patterns of small cylinders from individual choice-grade beef semitendinosus muscles, heated for several hours at 1°C intervals between 50 and 90°C is described. Beef semitendinosus muscle undergoes a marked decrease in shear, approximately one-half completed in 11 min at 58°C. This change in shear is a time-temperature rate process having a very high temperature dependence. Minimum shear values were obtained in the range of 60–64°C after heating for 30–60 min. In this time-temperature range the collagen shrinkage reaction is completed quickly while the hardening associated with higher heating temperatures is avoided. Relatively large differences, attributed to undefined biological differences, were noted in the shear versus heating time patterns for semitendinosus muscle cylinders from different animals.

INTRODUCTION

With few exceptions (Davies, 1956; Winegarden *et al.*, 1952) large pieces of meat such as roasts or steaks have ordinarily been employed for meat cookery investigation (Bramblett *et al.*, 1959; Ramsbottom *et al.*, 1945). Marshall *et al.* (1960) reported a temperature variation of approximately 60°C (108°F) within a single roast during the first few hours of cooking. In a relatively large piece of meat such as a roast, the time-temperature combinations to which any given point in the meat is subjected during cooking ordinarily vary widely from the surface to the center of the meat. It thus appears that small samples, in which heat transfer is rapid, are required to obtain definitive information on heat effects.

Changes in shear or tenderness produced upon heating are undoubtedly closely related to time-temperature-dependent protein heat-denaturation processes. Cover (1943) suggested that muscle fibers and connective tissue are the two structures in meat that contribute to its tenderness. Davies' work (1956) suggested, but did not demonstrate, that the initial decrease in shear produced on heating beef may be associated with the collagen shrinkage reaction. Ramsbottom *et al.* (1945) and Smith (1957) suggested that the decrease in tenderness of beef upon heating may be associated with coagulation of the muscle fiber protein.

The increase in tenderness achieved with long-time high-temperature heating is widely considered to be due to the collagen-gelatin transformation (Lowe, 1955). Thus there is reason to believe (although adequate evidence is lacking) that at least three major factors are involved in changes occurring during the cookery of meat. Little information is available, however, on the contribution of any of these factors to tenderness or the effect of time and temperature of heating on any of these factors. It is not known to what extent similar cuts from different animals differ substantially with respect to the effect of time and temperature of heating on shear.

Weir (1949), in a definitive work on the shrinkage of kangaroo tail tendon (used as a collagen model), found a heat of activation of 140,000 cal per mole. Winegarden *et al.* (1952) studied the shear of beef aponeurotic tissue strips heated at different times and temperatures. From the graphs of their results it appears that there is a

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difference of about 5°C between their shear results with the beef aponeurotic sheet collagen model and the shrinkage results of Weir (1949) for kangaroo tail tendon. Winegarden *et al.* (1952) interpreted their results as follows: "If the connective tissues in muscle are similar to the tissues in this study these results may be interpreted to mean that the cooking of steaks and roasts to the rare and medium done state brings about little if any change in the connective tissues of the muscles." According to Jensen (1949), rare beef is cooked to 60°C, medium beef to 71°C, and well done to 77°C. One objective of the present work was to find out if either of these models correspond to the marked time-temperature-dependent shear change observed when beef semitendinosus muscle is heated above about 58°C.

Veis and Cohen (1960) reported T_s values of 55.8–60.0°C for individual fibers of bull hide corium. According to Ritchey *et al.* (1963), "Whether the T_s of collagen in connective tissue within muscles is similar to that in skin of animals is not known."

It was desired to obtain an over-all picture of the effect of time and temperature on the shear of beef semitendinosus muscle with the thought that it might be possible to select experimental time-temperature ranges for singling out and relating shear changes to changes in specific protein structures in meat.

EXPERIMENTAL METHODS

Part A. Shear versus time patterns over the 50-90°C range. Frozen beef semitendinosus muscles of unknown history, from choice-grade rounds, were purchased as needed from local food supply channels and stored at −15°C. Semitendinosus muscle was chosen because it is one of the muscles from the round known to be the most consistent in tenderness throughout (Taylor *et al.*, 1961). Since it is a muscle of intermediate tenderness, it was thought that phenomena associated with both tender and less tender cuts might be discernible. This muscle yielded a relatively large number of samples, ½ in. in diameter by 2 in. long, making possible the replication necessary to minimize the effect of erratic readings reflecting large pieces of gross connective tissue in some samples. The requirement for a rapid rise in temperature was met by using cylinders ½ in. in diameter.

The Warner-Bratzler shear machine was used in obtaining an index of tenderness because of its wide acceptance in meat research and because it was considered impractical to use a taste panel in obtaining the large amount of information needed.

It was thought that studying the heating time effect at single temperatures would provide a more meaningful picture of meat cookery than studying temperature effects at single heating times. Thus, in most of this work, biological variables are largely eliminated in single temperature studies but are operative between temperatures.

To obtain the proper consistency of the meat and to facilitate the cutting of a maximum number of samples of uniform size, the individual muscles were partially thawed in a 4°C cold room for approximately 15 hr. Each muscle was cut into 45–70 cylinders, ½ in. in diameter and approximately 2 in. long. Essentially the entire muscle was used. Prior to heating, the samples were thawed completely, allowed to warm to room temperature, placed in 16-mm × 150-mm test tubes, and covered with 5-ml beakers. Samples were heated in a steam (coil) heated, temperature-controlled water bath held within ±0.5°C in this series of experiments.

Generally five or six cylinders were heated in test tubes for each of the 10–13 time intervals studied at a particular temperature. The test tubes were cooled 5 min in an ice-water bath and allowed to reach room temperature before shearing. Each test tube sample was sheared 3 or 4 times, and individual shear values (15–24 values) were averaged to obtain a single representative value corresponding to a single point on the time-versus-shear curves.

Between 50 and 70°C the heating experiments were generally carried out for 2 hr, and between 70 and 90°C the heating was carried out for 5 hr. Initially the 50–90°C temperature range was studied at 1°C intervals. Later the work was repeated at 5°C intervals in the same temperature range (50–90°C) for 5-hr periods to obtain confirmatory information. Seven runs were carried out at 80°C on beef from different animals.

In one experiment, heating time was held constant at 1 hr, and 6 cylinder lots (all from the same semitendinosus muscle) were heated at 23 different temperatures over the range of 50–90°C. Heating and shearing were carried out as in the above work except that the bath temperature was held to within ±0.1°C.

For study of the hardening reaction, heating was carried out for 120 min at 62, 67, and 72°C on cylinders from a single muscle.

The rate of heat transfer, for meat cylinders ½ in. in diameter and 2 in. long in 16×150-mm test tubes, was determined with iron-constantan

thermocouples embedded in the center of the meat cylinders. Measurements were carried out at 51, 70, 80, and 95°C. In all cases, irrespective of the starting temperature, the center of the meat cylinders came within 10°C of the bath temperature in 3½ min, and within 1°C of the bath temperature in 7 min after the tubes were placed in the water bath.

Part B. The effect of temperature on shear changes related to the collagen shrinkage reaction. Cylindrical samples (½ in. in diameter and approximately 1¼ in. long) were obtained from frozen beef semitendinosus muscles of good-grade rounds of unknown history. In this part of the work, 120–140 cylinders were obtained from each muscle.

Five experiments were carried out in which one half of the randomly selected cylinders from a single muscle were heated at one temperature and the other half were heated at a second temperature. In each case, a group of 5 or 6 randomly selected samples were removed periodically from the water-bath during the heating period of 50–60 min. In this part of the work the water-bath temperature was held within ±0.1°C. Several experiments were also carried out with single temperatures applied to meat from a single muscle. Temperatures below 56°C were too low, and above 59°C were too high, for study of the change in shear under consideration here, and shear values for temperatures outside this range were not used in the rate calculations given here.

To compare rates, the assumption was made that the change in shear was the result of a protein denaturation reaction and followed a first-order rate law. At the higher of the two temperatures used in any of the experiments, the shear change under study was essentially complete near the end of the heating time used. For rate calculation purposes the lowest average shear value occurring near the end of the heating period for the higher of the two temperatures was taken as representing the final shear value after completion of the change under study. Based upon the assumption of first-order behavior, the following equation was written (Weir, 1949):

$$S = (S_0 - S_\infty)e^{-k_s t} + S_\infty$$

S = shear at time t

S_0 = initial shear

S_∞ = final shear after completion of change

t = heating time

For estimating values of the reaction rate constant, k_s, log $(S - S_\infty)$ was plotted against time and k_s was calculated as -2.30 times the slope of the line. The method of least squares was used to locate the best line in all cases.

The Arrhenius constant, E_{Ar}, was computed by comparison of rate constants at two temperatures for three experiments. The average value of E_{Ar} was computed by using all of the available data for rate constants and plotting average log k_s values at specific temperatures versus the reciprocal of the absolute temperature $(1/T)$. The line was obtained by the method of least squares.

The values of the time for 1/2 of the shear change ($t_{1/2}$) at 56.0, 58.0, and 58.5°C were calculated from the rate constants obtained in the above manner using the equation

$$t_{1/2} = \frac{2.30 \log 2}{k_s} = \frac{0.693}{k_s}$$

RESULTS AND DISCUSSION

Part A. Shear patterns over the range 50–90°C. The shear values varied with heating time at different temperatures as noted in Fig. 1A-L. The average standard deviation of individual shear values from the mean of approximately 20 (15–24) shears for all of the work was ±2.77 lb force. The average standard deviation of the mean for the individual points plotted in Fig. 1A-L is ±0.62 lb. force.

The initial pH values of the meat used in this study, as observed with the Beckman Model G pH meter, were in the range of 5.34–5.54.

In line with the observations of Andross (1949), it was observed, in work not reported here in detail, that a substantial part of the loss of moisture associated with cooking the test-tube samples occurred within a few minutes at all temperatures employed (50–90°C).

In the range of 50–54°C (Fig. 1A,B) the shear pattern appears to be one characterized by high shear values and generally little change, although the 51 and 54°C samples exhibit divergent behavior.

In the 55–56°C range (Fig. 1C), change in shear due to the collagen shrinkage reaction becomes apparent. In the 57–59°C region (Fig. 1D) the shrinkage reaction is essentially completed in 1 hr. In the 60–65°C region (Fig. 1E, F) the shrinkage reaction is substantially completed after 15 min or less and there is little evidence of a hardening reaction in the shear pattern even after 5 hr of heating. In the range of 66–70°C hardening is substantial in ½–1 hr. This hardening reaction progresses rapidly

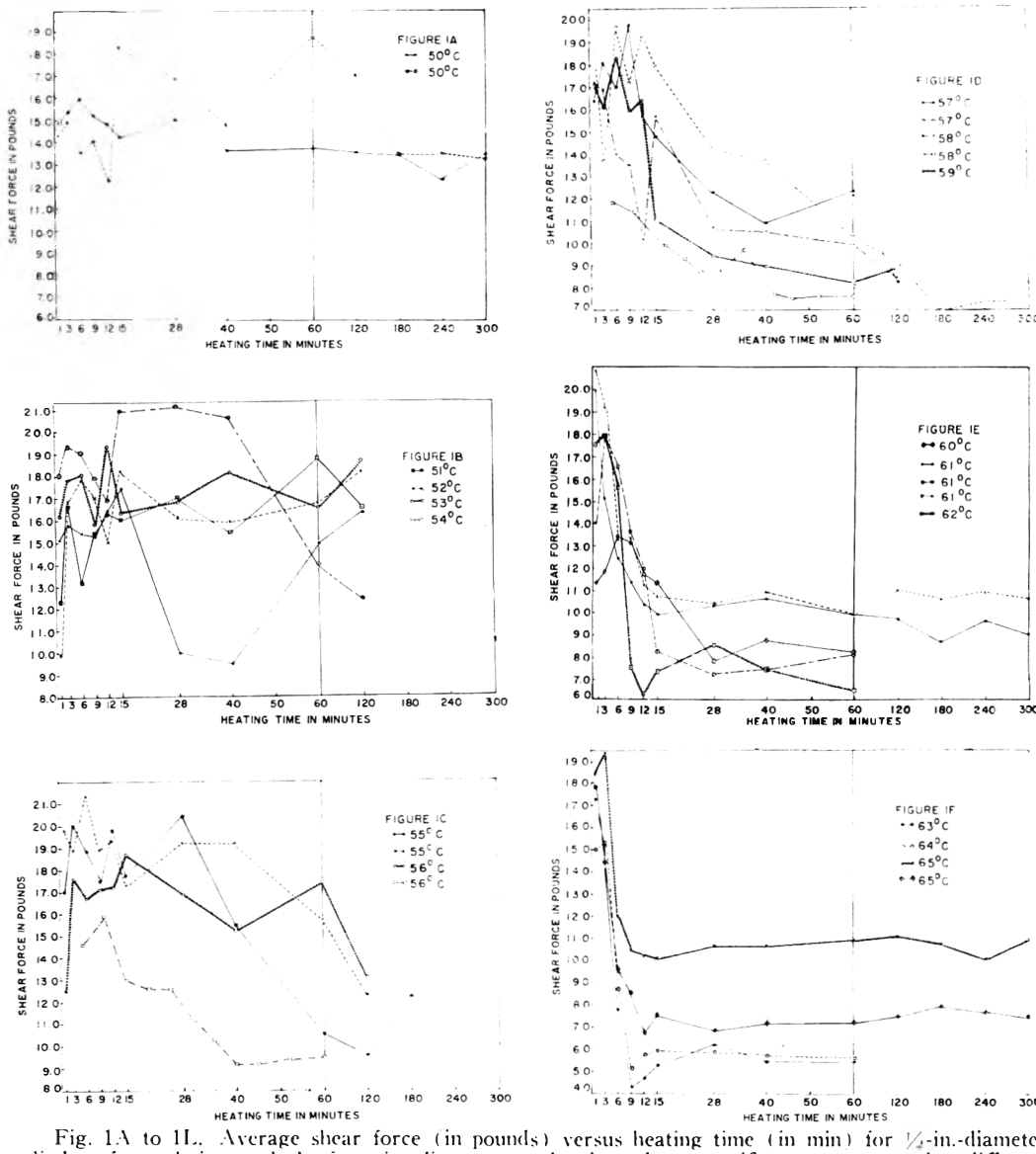
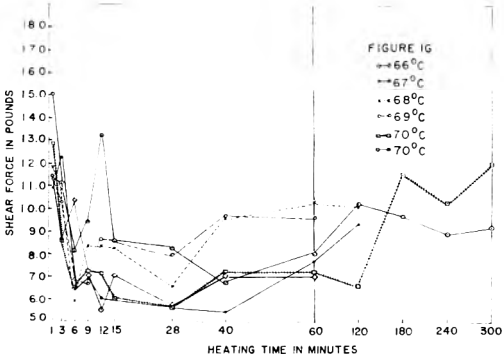


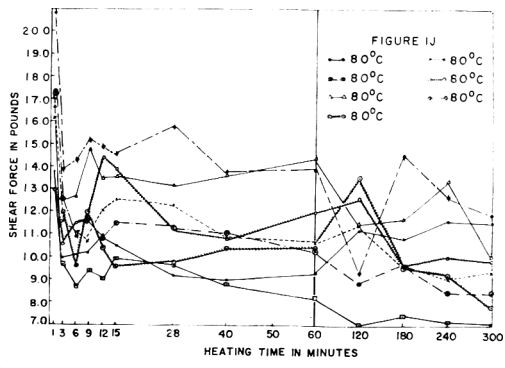
Fig. 1A to 1L. Average shear force (in pounds) versus heating time (in min) for ½-in.-diameter cylinders from choice-grade beef semitendinosus muscles heated at specific temperatures for different times.

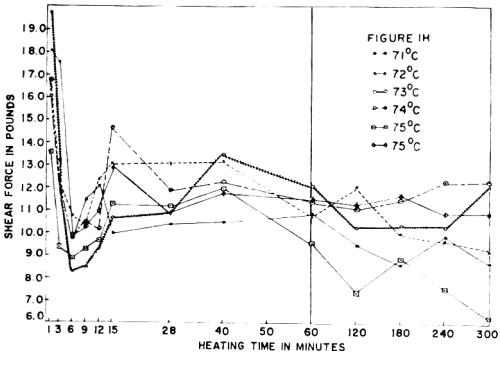
in the 71–75° range (Fig. 1I1) and appears to be substantially complete in about 30 min. This reaction may be associated with changes in muscle fiber proteins, though there is little evidence on this point either here or in the literature. Also, in the 71–75°C range as well as the 76–79°C range, a trend toward a decrease in shear after extended heating time is becoming evident, although not all samples exhibit such behavior.

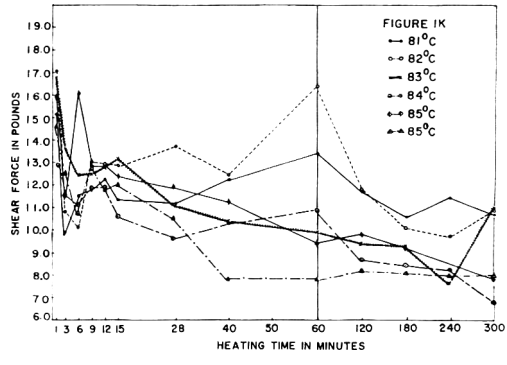
The results for 7 muscles heated at 80°C demonstrate that wide differences in behavior are to be expected in the meat from different animals. Further research to find biological reasons for such differences in behavior would be highly desirable.

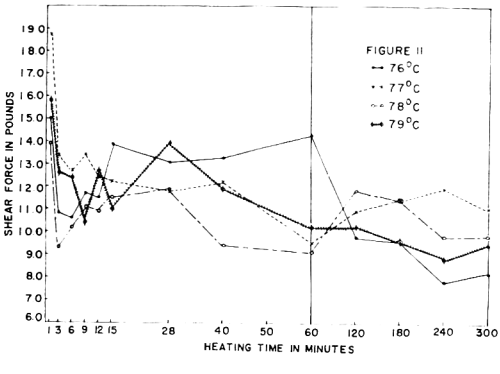
In the range of 80–90°C, collagen shrinkage and hardening are completed in a few minutes of heating. In this high temperature range, after a few minutes the shear values generally decline at a substantial rate during heating. The behavior of the 86 and 88°C samples is unexpected and un-

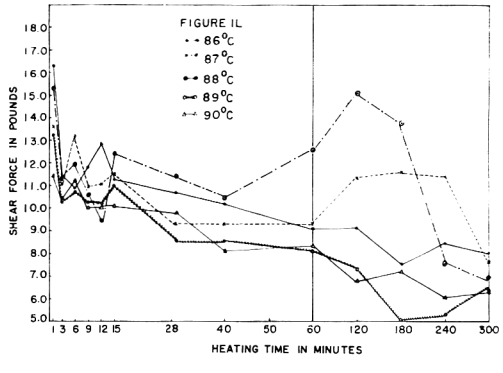












explained. It is likely that the major change exhibited during most of the heating time in this temperature range is the collagen-gelatin transformation, although further evidence should be developed on this point.

The results in Fig. 3 for cylinders from the same muscle heated at 62, 67, and 72°C are typical of those found in 3 replicate experiments. At 62°C little evidence of hardening is noted in 120 min, while hardening is complete in 80 min at 67°C and 30 min at 72°C. Since different proteins are likely precipitated at different temperatures it appears unlikely that the hardening reaction can be treated as a single rate process, as was the case with collagen shrinkage.

The fact that there is only a relatively small difference in shear over a very wide time period of 15 min to 5 hr in the 60–64°C range, indicates that it may be possible to minimize tenderness variables due to cooking time-temperature differences, in experimental meat cookery, by immersing

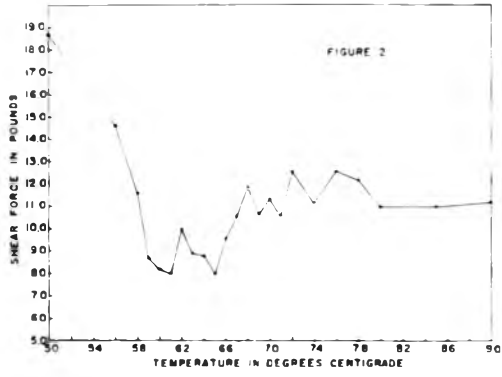


Fig. 2. Average shear force (in pounds) versus temperature (in °C) for ½-in.-diameter cylinders from the same choice-grade beef semitendinosus muscle heated 1 hr at 23 temperatures in the range of 50–90°C.

samples in a plastic bag in a water bath at 64°C for times long enough to ensure that the temperature of the slowest-heating portion is held in the 60–64°C range for about ½ hr or more. Further work is desirable, but results here suggest that it might be possible to achieve an evaluation of tenderness differences that actually represents biological differences, rather than differences introduced in cooking.

Finally, it should be emphasized that the results here were obtained with one type of beef muscle and that further work with other beef muscles or cuts would be required before broad generalizations could be made. Cover and Hostetler (1960) have pointed out that ". . . different cuts do not respond alike to any one set of cooking conditions."

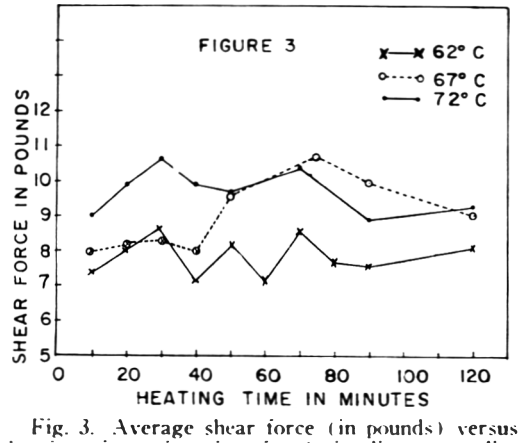


Fig. 3. Average shear force (in pounds) versus heating time (in min) for ½-in.-diameter cylinders from the same good-grade beef semitendinosus muscle heated at 62, 67, and 72°C.

Effect of temperature on shear change related to shrinkage reaction. Fig. 4 is typical of shear-versus-time data obtained in this work. In Fig. 5 the data of Fig. 4

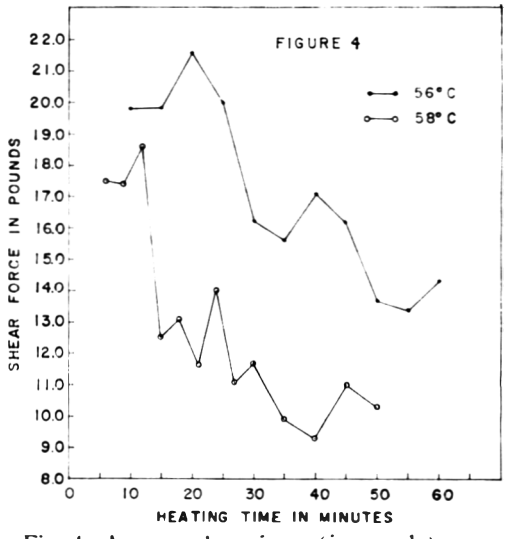


Fig. 4. Average shear force (in pounds) versus heating time (in min) for ½-in.-diameter cylinders from the same good-grade beef semitendinosus muscle heated at 56 and 58°C.

plotted as log (shear-shear$_x$) versus heating time are presented as a typical example of plots used to obtain rate constants. In this example the rate constants were 2.24×10^{-2} and 5.31×10^{-2} at 56 and 58°C, respectively.

Comparison of the present work, in which time for ½ of the change in shear ($t_{1/2}$) was computed to be 9.4 min at 58.5°C and 13.0 and 10.0 min at 58°C, with the approximately 9.5 min required for ½ shrinkage of kangaroo tail tendon at 60.1°C [as estimated by the present authors from Fig. 2 of Weir's paper (Weir, 1949)], indicates that the shear behavior in the present work differs from the shrinkage behavior of Weir's collagen model by approximately 2°C.

From the published curves of shear versus time of heating given by Winegarden *et al.* (1952) in their Fig. 2, for beef aponeurotic sheet, the present authors have estimated that at 65°C about ½ of the ultimate shear change occurred in approximately 11 min.

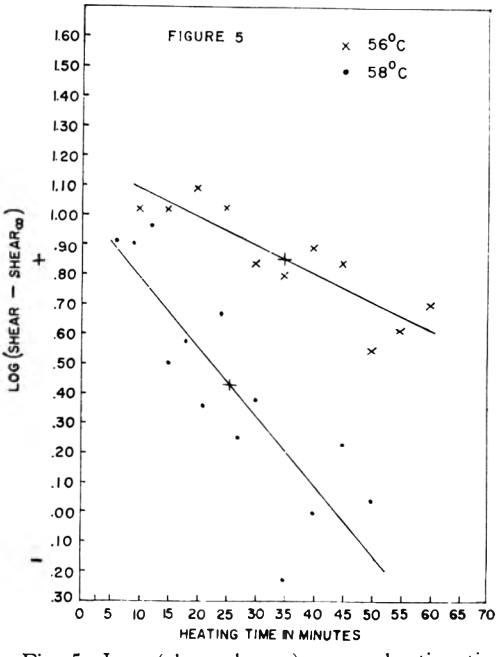


Fig. 5. Log (shear-shear$_\infty$) versus heating time (in min) for ½-in.-diameter cylinders from the same good-grade beef semitendinosus muscle heated different times at 56.0 and 58.0°C ($Y = 1.018 - 0.0213\ X$ for 58.0°C, and $Y = 1.18 - 0.0094\ X$ for 56.0°C).

It thus appears that the change in shear associated with collagen shrinkage is approximately 7°C lower in beef semitendinosus muscles than in the beef aponeurotic sheet model studied by Winegarden *et al.* (1952).

Although the error in the calculation of E_{Ar} is high in the present work (due to scatter in the shear values and the short temperature interval required to obtain results over a suitable time period), it is however, possible to estimate a value T_{sm} (temperature for ½ change of shear in 1 min) comparable to the collagen-shrinkage temperature value T_s [the temperature for ½ shrinkage in 1 min as defined by Weir (1949)]. At best, this comparison of present results with shrinkage temperatures of much of the work on collagen shrinkage can only be approximate since early workers ordinarily did not consider collagen shrinkage as a rate reaction.

Gustavson (1956) gives T_s values of 63–65° for calf skin and 65–67°C for cow skin. Borasky and Nutting (1949), using finely divided (60-mesh) cowhide collagen, observed rapid shrinkage at about 65°C. The average T_{sm} value for the shear change in beef semitendinosus muscle estimated from the present work is 63°C, with maximum values of 66° and minimum values of 61°C (Table 1). Weir (1949) found the heat of activation (ΔH) for the collagen shrinkage reaction in kangaroo tail tendon was 140,000 cal per mole.

Using the integrated form of the Arrhenius equation $\log k_s = -E_{Ar}/2.30\ RT + C$, the average Arrhenius constant E_{Ar} was obtained by plotting the data of Table 2 as $\log k_s$ versus $1/T$. The slope of the line (obtained here by the method of least squares) is equal to $-E_{Ar}/2.30\ R$. In this work the average Arrhenius constant E_{Ar} was estimated to be 141,000 cal per mole, using data from all of the experiments (Table 2). This very close agreement must be considered fortuitous considering the

Table 1. Kinetic data for the shear change associated with the collagen shrinkage from three paired-comparison experiments with beef semitendinosus muscle.

Muscle no.	Temperature	$k_s \times 10^2$ (min)	$t_{1/2}$ (min)	T_{sm} (°C)	E_{Ar} (kcal/mole)
1	56.0	2.24	31.0	61.0	94
1	58.0	5.31	13.0	62.0	
2	56.0	3.28	21.2	63.0	81
2	58.0	6.91	10.0	63.0	
3	56.5	1.30	53.4	66.0	188
3	58.5	7.37	9.4	66.0	
					Av 121

k_s = rate constant.
$t_{1/2}$ = time for ½ change in shear.
T_{sm} = temperature for ½ change in shear in 1 min.
E_{Ar} = Arrhenius constant.

Table 2. Rate constants for the shear changes occurring in good-grade beef semitendinosus muscle in the range 56.0–59.0°C.

Temp. (C°)	Av. $k_s \times 10^2$ (min)	N
56.0	2.86	2
56.5	1.30	1
57.0	2.86	4
58.0	7.28	2
58.5	7.37	1
59.0	12.0	1

scatter in the present data. For three experiments in which heating was carried out at two temperatures, E_{Ar} values were 94,000, 81,000 and 188,000, averaging 121,000 cal per mole (Table 1).

It is concluded from this work that the decrease in shear observable in the range of 56–59°C on heating beef is a manifestation of the collagen shrinkage reaction and that this change is a rate process having a very high temperature dependence.

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Techniques for Odor Measurement: Olfactometric vs. Sniffing

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SUMMARY

Two techniques of odor testing, olfactometric vs. sniffing, were compared with propionic acid used for the test odor. The olfactometer was more rapid and reliable as a technique for odor testing. Subjects appeared more sensitive by the sniff-method, but there were discrepancies in the data. The vapor phase above solutions of propionic acid in mineral oil was studied with propionic acid-1-C^{14}. Evidence is presented for the existence of non-ideal solutions. The significance of these data is discussed in terms of present techniques for odor testing, and their implications for future olfactory investigation.

The behavioral aspects of olfaction are difficult to investigate. Precise stimulus presentation is not easily accomplished, and subjects vary in their breathing patterns, thus altering the absolute concentration reaching the olfactory region. Even so, a controlled-blast method to give a constant sniff volume is an unreliable odor test since subjects may respond to the blast pressure itself when no odor is present (Wenzel, 1949, 1955). Therefore, other techniques were developed with no effort to control sniff volume, or use any non-normal form of breathing. Perhaps the most successful odor technique has been the sniff method. This test involves presentation of samples in beakers, test tubes, or jars for evaluation. The subject removes the cover and inhales the vapor above the solution. No elaborate equipment is required, and success has been considerable. The sniff method is used by The American Public Health Association as a standard test for the examination of odor quality and odor thresholds in water (1960).

Jones (1955a) reported a high degree of reliability for panel scores in absolute-threshold determination of *n*-butanol, safrole, and *n*-butyric acid in mineral oil. Comparing sniffing with the controlled blast, Jones (1955b) concluded that data were not directly comparable, because of differences in the breathing process. Other factors, however, might have accounted for the differences noted by Jones. The controlled-blast method uses only the purified test material, whereas the sniff method requires a diluent, mineral oil, benzyl benzoate, water, or some other inodorous, nonreactive material. Concentration in the vapor is calculated from the mole fraction and the perfect gas law, assuming that Henry's Law is obeyed in very dilute solutions. Unfortunately, this assumption has not been verified with evidence. Even so, much has been accomplished in the study of olfaction by sniffing. However, in recent years, there has been a renewed effort to develop olfactometers which control many of the variables influencing odor response and replace this sniff method (Wenzel, 1955; Mrak *et al.*, 1959; Johnston and Sandoval, 1960; Ough and Stone, 1961; and Stone *et al.*, 1962). These olfactometers are quite elaborate, but similar in that they are based on the principle of air-dilution. Briefly, a known volume of odor-saturated air is diluted in a larger volume of air, which flows by tubing to a subject seated with his head placed in a Plexiglas hood (Ough and Stone, 1961), or to a nose cone (Johnston and Sandoval, 1960). Data derived from one of these (Mrak *et al.*, 1959; Ough and Stone, 1961;

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Stone *et al.*, 1962) indicated that the instrument was reliable when air flow and temperature control were precise. Quantitative recovery of odor-saturated air and measurement of differential sensitivity were successfully accomplished as further evidence of its usefulness and reliability (Stone *et al.*, 1962; Stone, 1963). During those investigations, direct comparison of the two test methods (olfactometric vs. sniffing) was considered important in establishment of the olfactometer as a basic tool for further study of the olfactory process. Toward this end, determination of the 50% threshold for propionic acid was carried out with a single panel using the two test techniques. In addition, other comparisons were possible, including training, technique of presentation, replication, reproducibility, expense of operation, and psychological considerations.

EXPERIMENTAL

The compound chosen, propionic acid, was purified by standard chemical techniques, and purity was checked by gas-liquid chromatography with flame ionization. When not in use, the acid was kept in cold storage. While it is true that propionic acid may also activate other receptors on traveling to the olfactory region, for this investigation "response" is considered equivalent to "olfaction." A more apt term might be "nasochemoreception" (Moulton, 1963).

The solvent for the sniff test, mineral oil, chosen for its non-reactive, inodorous properties, has been used successfully by Jones (1955a, b) and others. It had an average molecular weight of 322 and a sp. gr. of 0.8676 g/ml (White Oil No. 3, Standard Oil Co., San Francisco). Sufficient mineral oil was purchased to ensure one source for the complete study.

Since sniffing is used to obtain a response in both test techniques, responses from the olfactometer are considered "olfactometric" for clarification purposes only.

The panel consisted of 3 male and 3 female undergraduate students, 18 to 23 years old. The students were paid an hourly wage as incentive to ensure completion of all tests, which were carried out daily in the morning, from Tuesday to Friday, for 30 minutes. Subjects had no previous test experience and were given 12 days of training at easily detectable levels to increase their confidence. The starting concentration for training was determined during selection of the applicants. These concentrations were decreased stepwise during training. For the olfactometer, final test concentrations were 0.42, 0.79, 1.6, 3.2, and 6.2 (all $\times 10^{-4}$ mg/L air). These concentrations were calculated from air flow rates, the vapor pressure of propionic acid (3.343 mm Hg at 26°C), and the experimental temperature. Previous reports had demonstrated that air sparged through a sample was saturated (Ough and Stone, 1961; Stone *et al.*, 1962; Stone, 1963), and these calculations were a valid method for determination of the odor concentration delivered to the subject.

The concentrations in the sniff method were determined from the mole fractions, assuming that Henry's Law was obeyed (0.39, 0.79, 1.6, 3.2, and 6.2, all $\times 10^{-4}$ mg/L air). Validity of the assumption of an ideal solution of propionic acid in mineral oil was tested by measurement of vapor-phase concentrations. This was done on solutions prepared with propionic acid-1-C^{14} (15 mc/mm, New England Nuclear Corp., Boston, Mass.) and the activity counted with a commercial scaler. The technique involved use of a sample flask containing the test solution with a sidearm attached to a Geiger Muller tube with a mica end-window (Stone, 1963). The sidearm was necessary to avoid counting stray-particle emission from the test solutions. The efficiency of this counting system was carried out by use of a standard source of known activity. From these data it was possible to determine counting efficiency of the system.

The forced-choice constant-stimulus method was the psychophysical method used (Guilford, 1954). Each day, half the subjects were tested first with the olfactometer, followed by the sniff method; the next day the sequence was reversed, i.e., order of test method was counterbalanced. Each subject received the same series of samples on day 1 with the olfactometer as was presented on day 4 with the sniff method. This was necessary to minimize learning and order effects. All tests were carried out at 26 ± 1.0°C. Subjects were presented with 11 test pairs at each test site, with no decision required on the first pair. The olfactometer test procedure was as described earlier (Stone *et al.*, 1962). Briefly, the subject was seated with his head inside a Plexiglas hood facing a scoreboard. A green light indicated no odor present, and a red light indicated odor present. Each sample of the pair was presented for 10 sec, with 15 sec between sample pairs. Previous investigation indicated no evidence of adaptation with a 15-sec interval between sample pairs. One sample of each pair was a blank; subjects knew only that some samples might be below their threshold. No blank pairs (Vexierversuche) were used, since one of each pair was a blank, although the subjects did not know this. Subjects were informed of the correctness of their decisions at the conclusion of each test period.

The olfactometer used in this study has been described in previous reports (Ough and Stone, 1961; Stone *et al.*, 1962). Briefly, a constant supply of conditioned and charcoal-filtered air is passed by appropriate valves and lines into the olfactometer. A portion of the air is by-passed through one of several calibrated flow meters to the test liquid into the diffusion bulb. The bulk of the air is passed through a large flow meter to a Plexiglas hood. The air metered into the diffusion bulb is saturated by passing through a sintered-glass sparger immersed in the test liquid. The saturated air is delivered back to the main air stream. Internal air temperature is maintained by a heating element installed in the main air stream with a manually-operated rheostat.

The sniff experiment was carried out in three partitioned, air-conditioned booths. Each subject received complete instruction, with emphasis on holding the lower half of the beaker, swirl gently, remove cap, and sniff. Samples, covered with tight-fitting aluminum caps, were tested in a predetermined order, with about 30 sec allotted per pair and no resniffing permitted, to duplicate the olfactometric procedure as closely as possible. After the test was completed, the order was randomized again for use by a second subject. Fresh solutions were added daily, an hour prior to testing. Preliminary investigation indicated about 20 minutes was required for the odor to saturate the vapor; therefore, the second subject was instructed to wait this period of time before starting the test. Although the solutions might have been used again, it was decided to discard them daily, since it is conceivable that a very dilute solution could be completely volatilized through opening for sniffing without the experimenter's knowledge. Sufficient test solution was prepared for a two-week test period and kept in cold storage until needed. This was necessary to prevent possible losses through contamination or unforeseen accidents.

RESULTS

The data were tested for homogeneity weekly and at the conclusion of the experiment. Data collected from the olfactometer were homogeneous ($\chi^2 = 2.9$), as shown in Table 1. Sniffing data were not homogeneous, as evidenced by $\chi^2 = 41.9$*** (5 df). As the experiment progressed, differences in sensitivity were noted for both techniques (Table 1). By the third week, sufficient olfactometric data were available for analysis, although it appeared that subjects had not yet reached their threshold by the sniff method. Therefore, two additional intensities were added to the sniffing series only, and at the conclusion of the experiment, these were tested separately to obtain sufficient replication for analysis.

Table 1. Weekly scores for the panel by both test methods.

Week	Correct	Total	% Correct
Olfactometer			
1	106	170	62
2	166	240	69
3	163	240	68
4	165	240	69
5	150	230	65
6	750[a]	1120	67
Sniffing			
1	148	170	87
2	199	240	83
3	155	240	65
4	257	336	76
5	235	322	73
6	62[b]	96	65

[a] $\chi^2 = 2.9$, $.50 > p > .70$.
[b] $\chi^2 = 41.9$, $p > .001$.
Sniffing (omitting week 3), $\chi^2 = 26.0$, $p > .001$.
Sniffing (omitting week 3 and 2 highest intensities), $\chi^2 = 25.2$, $p > .001$.

Analyses of the sniffing data for homogeneity yielded significant χ^2 values with or without the two highest intensities (Table 1) and week 3. Since the objective was comparison of the two test techniques, the sniff method was not repeated. These data were then analyzed by the "maximum-likelihood" solution (Jones, 1957), yielding tests of deviations from linearity and 50% threshold ± standard error of estimated (SE) as shown in Table 2. The regression analyses (Fig. 1) reflect

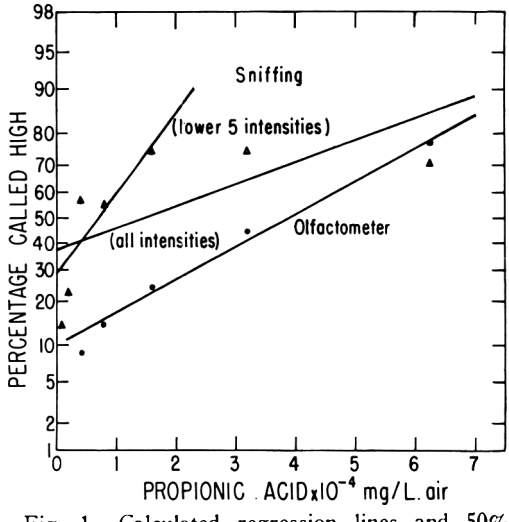

Fig. 1. Calculated regression lines and 50% thresholds for propionic acid by olfactometric and sniff methods, using 5 and 7 intensities respectively in the latter method.

Table 2. Analyses of regression equations of the lines and 50% thresholds for both test methods.

		Source of χ^2			
	df	Total 4	Regression 1	Residual 3	
Test technique		Sum of squares			χ^2_R	P
Olfactometer		0.3143	0.3117	.0026	0.5824	$.80 > P > .90$
Sniffing (5 lower intensities)		49.9824	33.7514	14.231	14.231	$.001 > P > .01$
	df	6	1	5		
Sniffing (all intensities)		68.0341	20.1189	47.9512	47.9512	$P > .001$

$\hat{Y}$ olf. $= [-1.2786 + .3319X]$ (10^{-4}).
$M_d \pm SE_{(M_d)} = 3.85 \pm .26 \times 10^{-4}$ mg/L air.
$\hat{Y}$ sniff. (5) $= [-.5519 + .8138\ X]$ (10^{-4}).
$M_d \pm SE_{(M_d)} = 0.679 \pm .206 \times 10^{-4}$ mg/L air.
$\hat{Y}$ sniff (7) $= [-.0521 + .1311\ X]$ (10^{-4}).
$M_d \pm SE_{(M_d)} = 0.897 \pm 1.526 \times 10^{-4}$ mg/L air.

the distribution of responses from the "sniff method." Both analyses of the sniffing method yielded highly significant χ^2_R terms, a result anticipated from the distribution of responses and heterogeneity of the data. Interestingly, the panel was more sensitive ($T_{50} = 0.679$ vs. 3.85×10^{-4} mg/L air) but less consistent by the sniff method. Regression analysis using all intensities in the sniff method was included to demonstrate the influence of the two highest intensities on the threshold value.

The results with propionic acid-1-C^{14} are shown in Table 3. These data indicate that the assumption of an ideal solution was not upheld, that the concentrations in the vapor state were much higher than predicted. Although it was not possible to measure every test concentration, these data indicate that samples were greater by a factor of 10^2 at 0.003 mg/L to about $10^{1.5}$ at the lower intensity. Application of these data to the 50% thresholds yields a value of 6.79 for the sniff method vs. 3.85×10^{-4} mg/L air for the olfactometer. The Critical Tables (Zwaardemaker, 1926) report a threshold value (corrected by 10^2) of 0.5×10^{-4} mg/L air for propionic acid; however, this represents absolute sensitivity, so that the thresholds reported here show reasonably good agreement.

Table 3. Calculated and observed concentrations of propionic acid-1-C^{14} in the vapor of mineral oil: propionic acid solutions.

Concentration (mg/L air)	
Calculated	Observed
.0003	.025
.00016	.0067
.000079	.00136
.000016	.00069

DISCUSSION

These data suggest a need for careful reappraisal of present odor-testing techniques, especially where a solvent system (diluent) is required. The experiments with propionic acid-1-C^{14} in mineral oil demonstrated the existence of non-ideal solutions, which were originally assumed to agree with Henry's Law. This unexpected deviation from the ideal may be due to the polarity of the propionic acid vs. the non-polarity of the mineral oil diluent; however, data are insufficient at present to warrant any definitive conclusion. Continued investigation of solvent systems may ultimately lead to the use of a series of solvents of varying degrees of polarity and non-polarity, depending upon the properties of the test odor. This would permit use of the sniff method for further study of olfaction on a quantitative basis. Benzyl benzoate has been used in the study of odor intensity (Beck *et al.*, 1954); however, it has a slightly aromatic odor and would not appear to be useful in quantitative investigations or study of differential sensitivity.

In spite of the concentration differences presented to the subjects, the two test methods can be compared from the standpoint of training, sensitivity, economy, technique of presentation, and psychological considerations.

Subjects appeared equally sensitive with both methods. The 50% thresholds, 3.85

Table 4. Individual responses according to the test method: olfactometric vs. sniffing.

Subject	Olfactometric method			Sniff method		
	Correct decisions	Total tests	% Correct	Correct decisions	Total tests	% Correct
1	103	190	54	183	238	77
2	125	180	69	178	224	79
3	134	190	71	183	238	77
4	129	190	68	160	238	67
5	139	190	73	194	238	82
6	120	180	67	158	228	69
Total	750	1120	67	1056	1404	75

$\times 10^{-4}$ (mg L air) for the olfactometer and 6.79×10^{-4} (mg L air) for the sniff method, are reasonably close when the latter is corrected according to the results of the isotope experiment. The difference can be attributed to the heterogeneity of the sniffing data, which is reflected in the standard error of estimate for the sniff method, $\pm$ 2.06. In terms of correct responses, Table 4 shows the individual scores according to the test technique. Subjects 3, 4, and 6 seemed equally sensitive under the two sets of conditions, whereas, 1, 2, and 5 were more sensitive by the sniff method. The greatest difference occurred with subject 1, whose responses were far below the panel average (54% correct, vs. 67% for the panel, $\chi^2 = 18.9^{**}$). This deviation from expectation by subject 1 may have been due to some factor(s) associated with the olfactometer. This is discussed under psychological considerations.

The possibility was considered that errors of the first or second kind were biasing the results (Baker *et al.*, 1954; Guilford, 1954). Evaluation of the data indicated that subjects were more inconsistent by the sniff method but that there was no trend by either method. There was less than 2% difference of incorrect decisions for the panel by either order of presentation, indicating that samples were presented in a random order and introduced no bias in the experiment.

Training was considered a factor of some importance. A 3-week period was thought sufficient to familiarize the panel with both techniques and stabilize their responses. The design (pair-comparison, constant-stimulus) was chosen for its simplicity, reducing the complexity of the subject's task. Observation of preliminary data had indicated that responses were homogeneous with both techniques. This conclusion was not corroborated by the heterogeneity of the sniff test data (Table 1). It is evident that the training period would depend on the nature of the test, and that there is no set time interval that can be uniformly followed. Olfactometric data were homogeneous, and data were sufficient for analysis by the third week. The study was continued in order not to bias the comparison with sniff test. It was interesting to note the differences in significance for the panel vs. the individuals in the olfactometric data. Since there were fewer than 50 tests per intensity (38) it was not possible to analyze these data further.

The speed of sampling in the sniff test was not controlled as well as with the olfactometer. It is conceivable that the panel's responses may have been less heterogeneous if samples were presented in a timed sequence; however, this was not possible in the present investigation.

In terms of cost, the sniff method is by far the most inexpensive test available: elaborate and costly air-flow and temperature-control systems are not required; beakers or bottles are readily available at little expense. Most diluents are not too expensive, and their cost would depend on the size of the study and the volume of the test solutions. The olfactometer does not require any diluent. Data are reported in quantitative terms, there is complete control of air flow and temperature, and immediate removal of odor. The olfactometer was designed primarily for study of the behavioral aspects of olfaction and other basic problems relating to the olfactory process. The present apparatus does not permit the use of

foods, and the study of mixtures requires more than one diffusion bulb, although an earlier report described a technique to study mixtures (Ough and Stone, 1961). Mixtures can now be studied by using additional diffusion bulbs (Ough *et al.*, 1962). The sniff method is more suited to odor quality-control or study of odor quality. Until problems of delivery of complex mixtures in the olfactometer are overcome, the sniff method will continue to be used for such studies.

Psychologically, the olfactometer would seem to be ideally suited for study of the olfactory process. The subject's task is simplified to pressing a signal button to indicate differences in concentration or intensity. Sample presentation is carefully controlled as to concentration and volume in the hood, and odor removal is not a problem with the constant-flowing purified air. However, the idea of placing one's head inside a Plexiglas hood may give rise to "anxiety" reactions or simply distract the subject enough to result in apparent decreased sensitivity. Subject 1 may be an example of the latter condition, since preliminary questioning of all subjects revealed no indication of claustrophobia anxiety, whereas in the sniff method the subject is confronted with a pair of coded beakers to be tested in a prescribed manner. Although 3-digit numbers are used, some subjects could conceivably have a bias that could influence response.

Removing the sample cover results in rapid dispersion of the odor throughout the test area. Odor fatigue may also be of some significance, especially in poorly-ventilated areas, although no evidence of this was noted here when as many as 16 test pairs were presented. Although no test samples were chromatographed to determine if the vapor contained propionic acid or possible contaminants, considerable care was taken to insure that purified propionic acid and mineral oil were used. The subjects noted that the propionic acid was easily recognized. It has been shown that as many as 100 samples can be presented to a subject in the olfactometer with no loss of sensitivity (Berg, 1963).

In conclusion then, it would seem that both methods have definite but dissimilar advantages and yield reasonably similar results. However, it is evident that each is better suited to a particular aspect of the study of olfaction. The sniff method is suited to study of odor quality, industrial quality control, and other investigations not involving quantitative investigation of the olfactory process, behavioral aspects, and odorimetry, for which the olfactometer is ideally suited. It may be possible to construct a less elaborate olfactometer; however, any loss in control of all possible variables might not be compensated.

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Relative Taste Intensities of Selected Sugars and Organic Acids[a]

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SUMMARY

A highly trained taste panel was used to establish the concentrations of fructose, glucose, and lactose equivalent in sweetness to sucrose at threshold and suprathreshold concentrations. The same panel established the sourness of lactic, tartaric, and acetic acids equivalent to that of citric acid at threshold and suprathreshold concentrations. There was no relation between pH, total acidity, and relative sourness. Results obtained from determinations made in water solutions agreed favorably with values reported in the literature. The threshold measurements are considered to be of limited value since the relative taste intensity of these compounds is not a constant but is materially affected by the absolute concentration chosen for comparison. The presence of a slight bitterness in glucose, and a slight, unidentifiable flavor in lactose, appeared to influence the threshold determinations. When the sweetness of sucrose and fructose were compared in pear nectar, fructose was less sweet than sucrose at all concentrations (1.0–20.0% sucrose). Increasing the total acidity of the pear nectar had little or no effect on the relative sweetness of these two sugars.

INTRODUCTION

Values reported in the literature on relative sweetness and relative sourness show marked lack of agreement, because of variations in testing methods, purity and concentration of the compounds, and sensitivity of the tasters (Fabian and Blum, 1943; Cameron, 1947; Warren, 1953; Nieman, 1960).

Since the sweetness of one compound relative to another changes with concentration (Dahlberg and Penczek, 1941; Cameron, 1947; Schutz and Pilgrim, 1957), it is generally agreed that comparisons of taste thresholds are unreliable for predicting relative taste intensity of supraliminal stimuli (Lichtenstein, 1948). However, few, if any, investigators have studied the magnitude of difference in sweetness and sourness between threshold and suprathreshold concentrations using the same panel of judges.

In general, relative taste intensities are determined in water solutions, and the assumption is made that the same values will apply in a more complex beverage or food. However, this hypothesis has not been tested directly.

The main objectives of the present investigation were to compare relative sweetness and sourness at threshold concentrations with those at suprathreshold levels, and to compare sweetness levels in water solutions with those in fruit nectar.

PROCEDURE

Panel selection and training. Using a paired method of presentation, a group of 26 people were tested daily for 15 days to determine their ability to distinguish differences in the sweetness of sucrose and in the sourness of citric acid. Six male and six female departmental employees, 23–48 years old, qualified for the final panel. One man and one woman dropped out of the study after completion of the sweetness determinations, so values reported for acid thresholds and relative sourness represent the responses of 10 judges.

All judging was done at 11 A.M. Monday through Friday in individual partitioned booths equipped with daylight illumination and maintained at 70±2°F. All solutions and nectars were at room temperature when tested. Stock solutions were made of all compounds (w/v), and final concentrations were prepared by dilution. Odorless freshly distilled tap water was used for preparing the solutions and for oral rinsing. All

[a] Presented in part at AAAS meetings, Denver, Colorado, December 29, 1961.

final water solutions were prepared 3–4 hr before tasting, and nectars were prepared 18 hr before tasting to ensure equilibration of sugars, which undergo mutarotation (Pangborn and Gee, 1961). Stocks and final concentrations were kept in glass-stoppered volumetric flasks.

Determination of taste thresholds. Thresholds for the eight compounds were established by the constant-stimulus paired-comparison method. Eight pairs of solutions, in which one member of each pair was always a distilled water control, were presented in randomized order at each session. Judges were instructed to circle the number of the sweeter, or more sour, sample within each pair. The compounds used, the range of concentrations presented, and the number of increments evaluated are listed in Table 1.

Threshold values were derived by plotting the panel's percent correct response to each concentration. The concentration at which 50% correct response was obtained was designated as the absolute threshold, whereas the concentration at which 70% of the responses were correct was the percentage required for significant identification at $p = 0.01$, as determined by χ^2 ($n = 80$).

Determination of relative sweetness and relative sourness. Concentrations of fructose, glucose, and lactose equivalent in sweetness to 0.5, 1.0, 2.0, 5.0, 10.0, and 15.0% sucrose were established by the constant-stimulus paired-comparison method, with sucrose at each respective concentration kept constant within each pair. Using the same method, acetic, lactic, and tartaric acids were compared with 0.005, 0.010, 0.020, and 0.040% citric acid, the latter's percentages being corrected for the one molecule of water of hydration.

Equivalent sweetness levels were obtained by plotting the percentage of the responses considering sucrose sweeter (or citric acid more sour) against the percent concentration of the compound being compared. Correlation coefficients and linear regression of these sets of data were calculated, and the concentration at which the regression line crossed the 50% response was designated as the equivalent sweetness or sourness. These equivalent values, in turn, were plotted against the sucrose or the citric acid percentages.

At a limited number of concentrations, depending on the solubility of the compounds, relative sweetness of the four sugars was established by the same technique, using canned pear nectar in place of distilled water. The nectar used for replication I consisted of 55% fruit and 45% water, whereas the nectar used for replication II consisted of 45% fruit and 55% water with no added sweetener. Composition of the nectars prior to addition of the sugars was:

	Nectar I	Nectar II
Soluble solids Determined by refractometry and expressed as ° Brix	8.48°	6.30°
Total acidity Expressed as citric acid anhydride	0.130%	0.102%
pH	4.10	4.26

To test the effect of apparent sourness on the relative sweetness of fructose and sucrose, two additional studies were conducted on pear nectar II; comparisons were with nectars containing 0.1% and 0.2% added citric acid.

RESULTS AND DISCUSSION

Relative sourness. Values representing concentrations of equal sourness for the four organic acids are shown in Table 2. At threshold concentrations, citric acid was considered to be the least sour and tartaric acid the most sour of the four compounds. The magnitude of this relationship is seen more readily in Fig. 1. At suprathreshold concentrations, citric acid, once again, is the least sour of the four acids, but there was little if any difference in the sourness of the other three acids (Fig. 2). At these concentrations, lactic acid showed a linear rela-

Table 1. Compounds used in threshold studies.

Compound	Source	Range (%)	Increments[a]
Sucrose	Baker, #23617	0.010 –0.171	9
D-Glucose (anhydrous)	Eastman, #84	0.009 –0.720	10
Fructose	Eastman, #679	0.010 –0.040	9
Lactose	Baker, #91374	0.036 –0.360	6
Citric acid	Baker-Adamson, #1063	0.0001 –0.0038	7
Acetic acid	Baker, #20518	0.00006–0.0024	10
Tartaric acid	Baker & Adamson, #1203	0.00015–0.0030	7
Lactic acid	Baker, #20722	0.00014–0.0027	7

Table 2. Relative sourness of four organic acids.

% correct response	Citric acid (MW = 192.1)		Acetic acid (MW = 60.1)		Tartaric acid (MW = 150.1)		Lactic acid (MW = 90.1)	
	%	M	%	M	%	M	%	M
Average threshold concentrations $\times 10^{-4}$								
50 [a]	2.0	0.104	1.2	0.200	1.5	0.100	0.9	0.100
70 [b]	8.0	0.417	6.0	1.331	2.2	0.146	3.8	0.422

Citric acid			Acetic acid			Tartaric acid			Lactic acid		
pH	%	M	pH	%	M	pH	%	M	pH	%	M
Average suprathreshold concentrations of equal sourness $\times 10^{-4}$											
3.64	50	2.6	3.62	40	6.7	3.97	40	2.7	3.69	40	4.4
3.40	100	5.2	3.36	80	13.3	3.81	90	6.0	3.54	80	8.9
3.21	200	10.4	3.16	170	28.3	3.66	160	10.7	3.26	180	20.0
3.00	400	20.8	2.98	310	51.6	3.50	310	20.7	3.08	350	38.8

[a] Absolute threshold (concentration at which 50% of the responses correctly distinguished the solution containing acid from a distilled water blank).

[b] Represents the % correct response required for significant identification of acidified samples at $p = 0.01$.

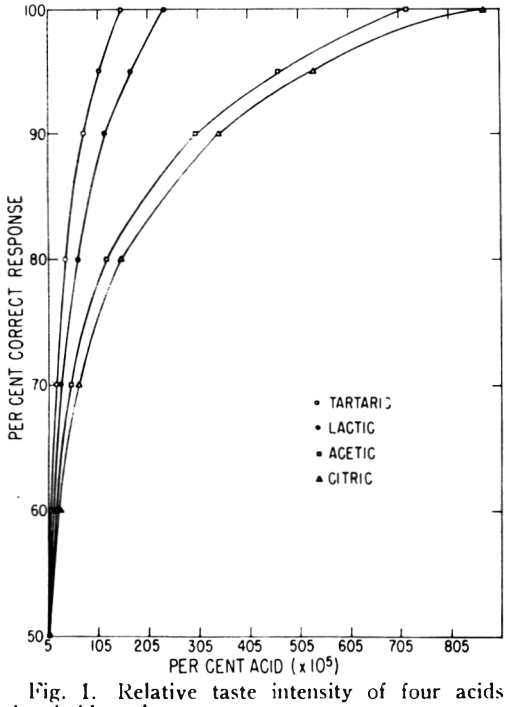

Fig. 1. Relative taste intensity of four acids; threshold study.

tionship to citric, whereas tartaric and acetic fit slight curves. It should he pointed out that in the determinations of relative intensities at threshold and at suprathreshold concentrations, two distinct psychophysical tasks confront the experimental subjects, e.g., in the threshold studies, subjects used any and all sensory clues in distinguishing between distilled water and low concentrations of the acid; at suprathreshold levels where all samples are distinctly sour, difference in intensity of sourness guides the subject's choice. Concentrated solutions of acetic acid possess a distinct, characteristic odor, which Harvey (1920) described as "somewhat aromatic"; at the concentrations used in the present study, however, no one commented on any differences in the odor properties of any of the solutions. Nonetheless, it is possible that subliminal olfactory clues may have contributed to the large differences observed in the response to the four acids in the threshold studies. This would suggest that the nose should be

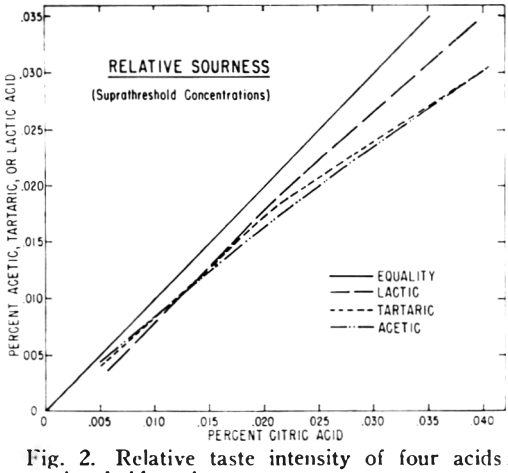

Fig. 2. Relative taste intensity of four acids; suprathreshold study.

closed when only taste comparisons are sought.

The threshold values obtained for these four acids are considerably lower than the median values cited by Pfaffmann (1959) and by Fabian and Blum (1943). The citric acid thresholds, however, compare favorably with determinations made in this laboratory previously (Pangborn, 1959) where an average value of 0.00095% was obtained after six exposure periods. The threshold values determined by Berlatzky and Guevara (1928) for lactic, citric, acetic, and tartaric acids were respectively 0.09%, 0.08%, 0.075%, and 0.07%. Fabian and Blum (1943) list taste thresholds of 0.008%, 0.011%, 0.012%, and 0.013%, respectively, for lactic, tartaric, acetic, and citric acids. According to the summary of the literature prepared by Pfaffmann (1959), the order was tartaric, acetic, lactic, and citric. Ough (1963) tested four food acids at suprathreshold levels in wine and observed the following relative sourness, in decreasing order: tartaric, citric, fumaric, and adipic.

In general, the sour taste is associated with the hydrogen ion and, to a lesser extent, to the degree of dissociation. Strong acids (fully dissociated) are more sour than equinormal solutions of a weak acid. The present data show that, at equivalent sourness levels, tartaric has the highest pH whereas acetic has the lowest. Crozier (1916) suggested that the potentially ionizable hydrogen is a factor influencing sourness as well as the actual hydrogen ion concentration. Harvey (1920) reported an interdependence of sourness, hydrogen ion concentration, and the total free acid of the solution. Baráth and Vándorfy (1926) showed that hydrochloric acid solutions were more sour than equinormal solutions of acetic acid except at concentrations near the threshold. These investigators concluded that the greater sourness of a weak acid over a strong acid at the same pH was due to the greater "potential acidity" of the former. Based upon the comparisons made by one experienced taster, Beatty and Cragg (1935) concluded that sourness was equal to the volume of phosphate buffer solution required to bring a unit volume of the acid to pH 4.4, divided by "K," a constant characteristic of the buffer used:

$$S = \frac{X}{K}$$

If the foregoing theory is correct, the buffering capacity of the saliva bathing the taste receptors could influence the hydrogen-ion concentration as well as the dissociation constants of the test acid in the mouth. In electrophysiological studies made by Beidler (1957), equal afferent nerve discharges were not achieved with equal pH, normality, or molarity. It is apparent, therefore, that some basic peripheral and/or cortical physiological mechanism complicates the relation between sourness and the chemical and physical properties of acids.

Relative sweetness. Responses to the sweetness of the four sugars at threshold concentrations are plotted in Fig. 3. As expected, sucrose was sweeter than glucose and lactose, and less sweet than fructose. Plotting the responses in this manner dramatically illustrates the differences in taste properties between sugars compared against water instead of directly. Sucrose and fructose elicited the sweet taste only. Glucose elicited sweetness, and at higher concentrations, a slight bitter aftertaste was perceived.

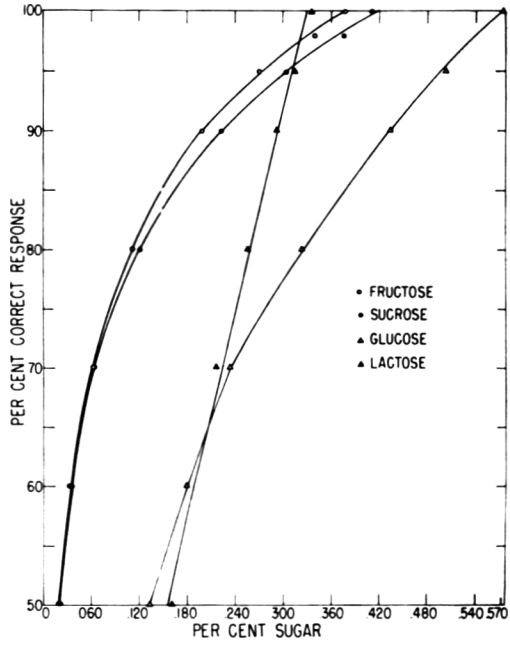

Fig. 3. Relative taste intensity of four sugars; threshold study.

Similar observations were made by Cameron (1947). Qualitative changes in glucose at higher concentrations have been reported by other investigators also (Lichtenstein, 1948; Schutz and Pilgrim, 1957). Further work is needed to establish whether this bitterness is a property of the glucose molecule or due to an impurity that may be present in minute quantities.

In the determinations of the threshold for lactose, the panel recorded a fleeting "fruitiness" in solutions above concentrations of 0.25% lactose. This latter property of the lactose solutions distinguished them from water more readily than sucrose was distinguished, thereby giving the impression, when plotted in Fig. 3, that lactose was sweeter than sucrose. It is possible that lactose, which is used by the chemical and pharmaceutical industries as an absorbant of riboflavin and certain pigments, picked up flavors from its environment and imparted them to the taste solutions. Therefore, a new supply of lactose was obtained (Foremost Dairies, San Fancisco), and thresholds were retested, using the six remaining subjects from the original study. This lactose could not be distinguished by odor or by taste from samples that had been heated with charcoal and recrystallized. Sweetness responses were determined for solutions made from the untreated lactose, and from lactose that had been heated 30 min in a single-stage vacuum oven at 55–58°C, a treatment that should have been sufficient to drive off any volatiles present. These results are shown in Table 4. Plotting these figures, determining the lines of best fit, and extrapolating back to the 50% level of response did not prove useful, for the values so obtained were respectively −0.33 and −0.23% lactose for the heated and unheated samples. Using a linear-probability scale described by Jones (1957), where concentration is plotted against percent correct response above the chance level, the "75% correct" threshold was calculated at 0.89% (0.025*M*) lactose. Alternatively, calculations were made of the concentrations that gave a significant number of correct responses on the basis of 48 individual tastings per concentration. As shown in Table 4, these values (at $p = 0.01$) for heated and unheated lactose were respectively 0.52 and 0.61%, which is considerably higher than the 0.22% reported by the original panel (Table 3). The responses to sweetness in the second set of determinations are nowhere of the same magnitude as those of the original investigation. The following factors, listed in their order of importance, may have combined to account for this discrepancy: 1) the second sample of lactose had a greater purity and contained no "fruity" flavor; 2) only six of the original twelve subjects were available for retesting; 3) there may have been loss of sensitivity due to the time interval between the two sets of determinations. However, the latter set of data (Table 4) are considered more reliable in terms of true sweetness responses to lactose than the former (Table 3).

At suprathreshold levels, values obtained from determinations of relative sweetness in water solutions agree very well with figures reported previously (Table 3). For example, Cameron (1947) found that 5% sucrose was equivalent in sweetness to 4.6% fructose, 8.3% glucose, or 14.9% lactose; corresponding figures reported by Dahlberg and Penczek (1941) were 4.5%, 7.2%, and 13.1%. Plotting the concentrations of fructose, glucose, and lactose (purified sample) equivalent in sweetness to the seven concentrations of sucrose gives a straight line for fructose and a smooth curve for the other sugars, similar to Cameron's (1947). Using a method of paired comparison, Lichtenstein (1948) observed a similar relationship when glucose and sucrose were compared. With a single-stimulus intensity-rating method, Schutz and Pilgrim (1957) observed that mean ratings of subjective sweetness intensity plotted against concentration for 15 compounds yielded relatively smooth curves, such that logarithmic spacing provided nearly straight lines.

When the sweetness intensities of fructose, and glucose relative to sucrose were determined in pear nectar I, it was noted that sucrose was considered sweeter than fructose at all concentrations and the relative sweetness of glucose was less than it had been in water (Fig. 4). The supply of pear nectar I was exhausted, necessitating

Table 3. Relative sweetness of four sugars.

% correct response	Sucrose (%)	Fructose (%)	Glucose (%)	Lactose (%)
I. Threshold concentrations. Values represent average panel threshold				
50 [a]	0.017	0.016	0.132	0.160
70 [b]	0.064	0.059	0.242	0.220

	% fructose					% glucose			% lactose
				Pear nectar II					
% sucrose (standard)	In water	In pear nectar I	In pear nectar II	+ 0.1% citric	+ 0.2% citric	In water	In pear nectar I	In pear nectar II	In water
II. Suprathreshold concentrations. Values represent concentrations of equal sweetness									
0.5	0.42	...	...	...	...	0.89	...	...	1.90
1.0	0.76	...	1.09	1.15	1.10	1.84	...	1.74	3.46
2.0	1.66	2.29	2.20	2.28	2.32	3.57	3.76	3.35	6.54
5.0	4.19	5.38	5.42	5.46	5.74	8.28	8.35	8.08	15.74
10.0	8.62	10.54	10.53	10.22	10.72	13.86	15.38	14.02	25.92
15.0	12.97	15.62	14.94	15.24	15.32	20.00	...	20.15	34.60
20.0	15.44 [c]	21.10 [c]	19.58	20.52	20.20	...	...	24.80	...

[a] Absolute threshold (concentration at which 50% of the responses correctly distinguished the solution containing sugar from a distilled water blank).

[b] Represents the % correct response required for significant identification of sweetened samples at $p = 0.01$.

[c] By extrapolation.

Table 4. Sweetness of solutions prepared from heated and unheated lactose.

Concentration		Percent correct identification	
		Unheated lactose	Heated lactose
%	*M*	(n = 48)	(n = 48)
0.072	0.002	52.1	52.1
0.216	0.006	58.3	64.6
0.288	0.008	66.7	62.5
0.360	0.010	64.6	68.8
0.721	0.020	68.8	81.3
1.08	0.030	81.3	77.1
1.44	0.040	85.4	83.3
Correlation coefficient		+0.96	+0.88

Concentrations at which correct responses were significant at:

	%	*M*	%	*M*
p = 0.05	0.43	0.012	0.32	0.009
p = 0.01	0.61	0.017	0.52	0.014
p = 0.001	0.89	0.025	0.84	0.023

using pear nectar II, which was more dilute. The response to glucose could not be repeated in pear nectar II, but fructose showed the same pattern, i.e., being less sweet than sucrose. It was speculated that the acidity of the pear nectar may have affected the sugars differentially, either by reduction of apparent sweetness or by influencing the hydrolysis of the sucrose.

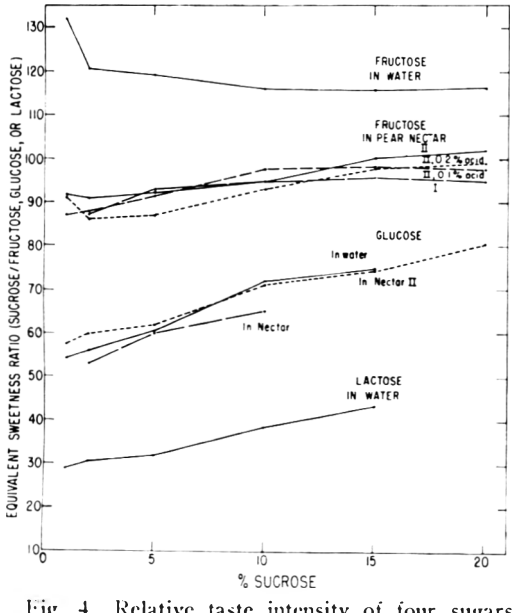

Fig. 4. Relative taste intensity of four sugars in water and in pear nectar; suprathreshold study.

Therefore, the comparisons were redone in pear nectar at two levels of added citric acid. Previous studies with water solutions had indicated that the sweetness of fructose was reduced by citric acid to a slightly greater extent than was the sweetness of sucrose (Pangborn, 1963). However, as shown in Fig. 4, the acidity of pear nectar was not responsible for the discrepancies observed between water solutions and pear nectar. Perhaps the differences in the "apparent activity" (associative tendencies, intramolecular hydrogen bonding, water-solvent binding properties, etc.) between sucrose and fructose influenced the values obtained. The viscosity of the nectar may have had a differential effect on the two sugars. Although there is an apparent relationship between relative sweetness and relative solubility of the four sugars studied, solubility *per se* is not believed to be of importance, since all compounds were dispersed in the solutions prior to tasting.

The observation of differences in relative intensity between aqueous solutions and a natural food product, nectar, suggests that assuming that complex foods will have the same relative sensory properties as do model systems, can be erroneous.

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Variability of Taste Perception[a]

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SUMMARY

High inter-personal variability has been noted in the taste qualities people report for solutions of sodium benzoate. This study sought to determine whether such variability occurs with other substances and to assess the role of learning in development of the response. 24 *S*'s replicated ratings of the intensity of *sweet*, *salt*, *sour*, and *bitter* in solutions representing 3 concentrations each of 4 familiar and 4 unfamiliar substances. Significant interpersonal variability was found for all substances. It was lower for the familiar substances (sucrose, salt, citric acid, and caffein) than for the unfamiliar ones (sodium benzoate, monosodium glutamate, potassium chloride, ammonium chloride, and a "model" solution). Thus, learning appears to be important.

Sodium benzoate, during a period starting about 9 years ago, created a flurry of interest among those concerned with the sensory evaluation of foods. A. L. Fox (1954) suggested, on the basis of some extensive experimentation, that people's taste responses to this chemical could reliably classify them as *sweet*, *salt*, *sour*, *bitter*, or *tasteless* tasters. People grasped at this straw of information. Here was a bold new way to evaluate people and perhaps to select taste panels. However, interest gradually waned—in large part because no one was able to verify Fox's results (Hoover, 1956).

The Food & Container Institute conducted a series of experiments directed toward the effects of sodium benzoate itself. The results failed to confirm Fox *in toto*—in fact, they suggested that he was more wrong than right; however, they did verify his finding of high interpersonal variability in the qualities that people use to describe the taste of sodium benzoate (Peryam, 1960). Evidence against Fox's theory included: 1) there were important sources of variability other than inter-personal; 2) people typically responded with multiple rather than single qualities; and 3) they could not be classified reliably on the basis of their responses. It was conjectured that much of the variation was probably related to differences among people in learning and language, and, if so, that it would not be unique to sodium benzoate.

The studies reported here were undertaken in an attempt to determine the degree of generality of the sodium benzoate effect. They were extensive, and only selected aspects of the results are reported. Focal points of the investigation were the following hypotheses:

1) Other substances will show the same kind of taste response variability as does sodium benzoate.

2) Variability will be greater for substances that are relatively unfamiliar to the test subjects than for substances that are familiar.

3) Certain features of a given person's responses will tend to be common among unfamiliar substances, but will be less so among familiar substances.

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This research was undertaken at the Armed Forces Food & Container Institute and has been assigned number 2198 in the series of papers approved for publication. The views or conclusions are those of the author, and are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

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Hypotheses 2 and 3 derive from the assumption that learning plays a major role in determining variability.

METHOD

Substances selected for investigation were: sodium chloride, sucrose, citric acid, and caffein, to represent familiar tastes; and sodium benzoate, potassium chloride, ammonium chloride, monosodium glutamate, and a "model solution," to represent unfamiliar tastes. The "model solution" was a mixture of the four familiar substances in proportions selected so that the intensities of *salt*, *sweet*, *sour*, and *bitter* should have been equal. All chemicals were C.P. grade or equivalent. Solutions were prepared with fresh, charcoal-filtered distilled water. A stock solution was prepared and diluted on a volume basis to assure accurate measurement of low percentages. Samples, 1 oz in quantity, were presented in whiskey shot glasses. All testing was done in special test booths in the Sensory Evaluation Laboratory at the Food & Container Institute.

Fig. 1 illustrates the test method. Instructions to the subjects were, essentially: "Taste the sample, re-tasting as often as you consider necessary, and rate the intensity of each of the flavor qualities, considering them in the order shown on the questionnaire." Subjects rinsed their mouths and waited 1 min between samples.

The test subjects were recruited from a pool of volunteers who regularly participate in food preference tests. The only selection criteria were willingness to test and availability for the entire series of tests. The panel was primarily male, the modal age was 22, and only a few had ever participated in taste experiments other than the rating of food preferences. Each subject participated in one or two training sessions to assure understanding of the task and of the test procedure.

A series of 3 solutions—"weak," "medium," and "strong"—was developed for each substance, and these series were equated for intensity among substances by an experimental procedure. The actual concentrations used are shown in Table 1. These 27 solutions, plus 3 distilled-water blanks, constituted the series for the flavor analysis experiment. A random order of the 30 samples was established for each of the 24 subjects, and the samples were tested, 5 at a session, in 6 sessions on different days. The order in which the four qualities appeared on the questionnaire was systematically varied. The entire experiment was replicated, using a new random order for each subject.

For analysis, the values 0-8 were assigned to the points of the scale, beginning at the "none" end. Analyses of variance were made on the data, assuming a Model I (Brownlee, 1960), where the sample is regarded as consisting of all groups in the population, and using replicate error as the error term. Separate analyses were done on each of the substances, including water.

Table 1. Percent concentration of solutions used in the flavor analysis.

Substance	Concentration level		
	Low	Medium	High
Sucrose	.453	1.980	8.600
Citric acid	.006	.022	.081
Sodium chloride	.041	.177	.770
Caffein	.014	.058	.241
Sodium benzoate	.120	.737	4.512
Potassium chloride	.043	.196	.897
Ammonium chloride	.015	.056	.210
Monosodium glutamate	.026	.226	2.017
"Model" solution	6.250	19.300	59.500
Constituents:			
Sucrose	.325	1.004	3.094
Sodium chloride	.035	.108	.333
Citric acid	.003	.010	.031
Caffein	.005	.015	.048

RESULTS

Figure 2 is an empirical display of the results. For each of the 9 substances (excluding water) the average intensity of each quality, based on 48 responses, is shown as a function of log concentration. In examining these charts it should be remembered that the over-all intensities were the same for all substances.

Sucrose and sodium chloride present clear pictures. The appropriate quality, *sweet* or *salt*, rises to a relatively high level, whereas the other three qualities stay near the zero line. With citric acid and caffein, the expected quality is clearly dominant, but some confusion is shown. *Bitter*, with citric acid, and *sour*, with caffein, also rise above the negligible level.

Inspection of the curves for sodium benzoate suggests that *sweet* is the dominant quality, but note that *bitter* rises to a higher level at the highest concentration, also that both *salt* and *sour* come into play. It is emphasized that these are averages for 24 people. The curves definitely show multiple qualities, but not whether this is because each subject perceived multiple qualities or because people just disagreed.

Potassium chloride is generally recognized as tasting *bitter* and *salt*, and ammonium chloride as *bitter* and *sour*. The charts agree, but also show that two qualities are not enough to describe

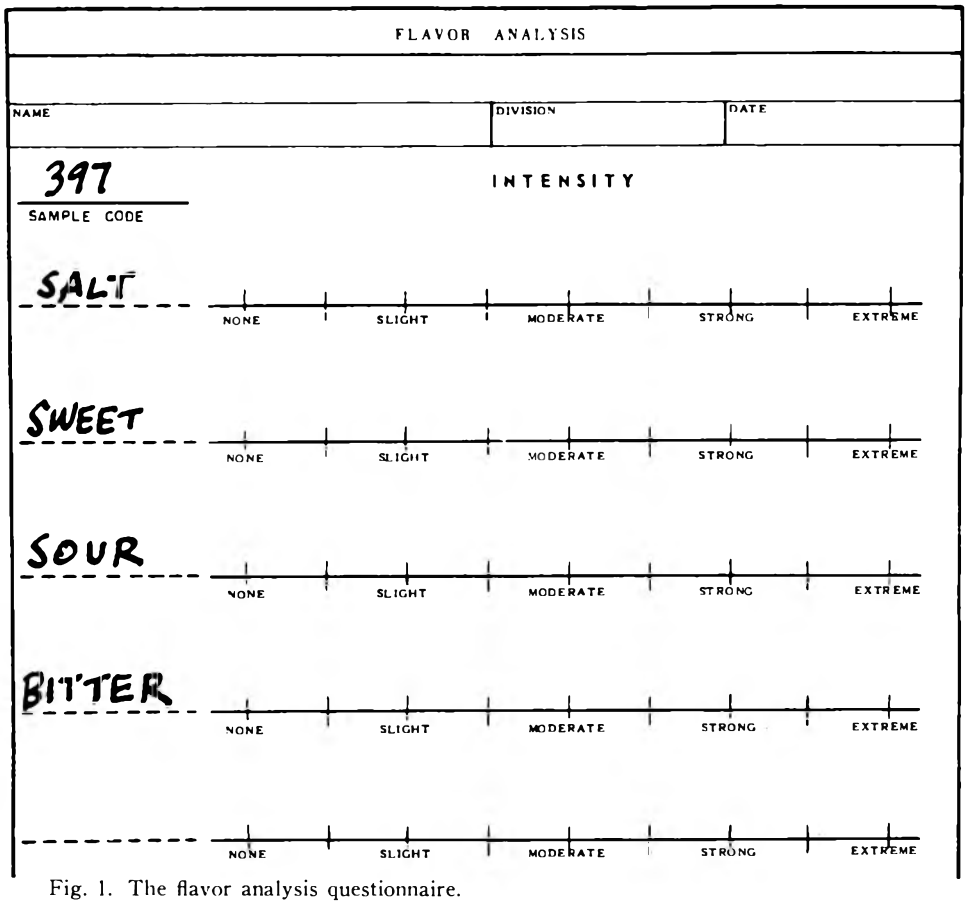

Fig. 1. The flavor analysis questionnaire.

either of these substances; potassium chloride has an important component of *sour*, and ammonium chloride shows *salt*. Only *sweet* fails to appear.

Monosodium glutamate appears to cause even more disagreement than does sodium benzoate. *Sweet* stays relatively low, whereas the other three qualities all show significant intensity and are about equal.

The data on the "model solution" seem to demonstrate the failure of the objective in using this approach. *Sweet* is the dominant quality and increases progressively although *salt* emerges at the highest concentration. It is possible that the sucrose concentration was simply too high; on the other hand, there is some evidence in favor of the intrepretation that the dominant position of *sweet* in a mixture where all four qualities should have been about equal may have been a contrast phenomenon.

Table 2 presents an analysis of the replicate error. Each subject tested each solution twice, and this gave a component of variance that could be partialed out according to any of the other variables. The table shows the average for each level of each of the four independent variables. Note the wide range and rather flat distribution of error across subjects. The ratio was 7:1 between the largest and smallest variance. Some consideration was given to eliminating the highly variable subjects; however, analysis showed that the distribution did not depart significantly from normality.

Replicate error varied considerably as a function of substance. The smallest error (0.6) for water was less than one-third of the highest error (2.0) for monosodium glutamate. The low value for water is favorable evidence of stability of judgment. The four "familiar" substances (sugar, salt, caffein, and acid) plus water show the smallest error, and there is a definite cut-off between these and the "unfamiliar" substances.

Error varied regularly according to solution concentration, with error for "high" being over twice that for "low." Part of this difference is probably "real" in the sense that people can judge moderate and weak stimuli more accurately; however, it is also likely that part of the effect is an artifact of the scaling method. As actual

intensity approaches zero, and ratings approach the lower end of the scale, the opportunity for variation in one direction is denied, so apparent variation is reduced.

Comparison of replicate error for the four qualities reveals clear differences. The relative order of precision of judgment is *sweet, salt, sour, bitter,* with the error for *bitter* nearly twice that for *sweet.* These differences were not due to scale effect, as were the concentration differences, since

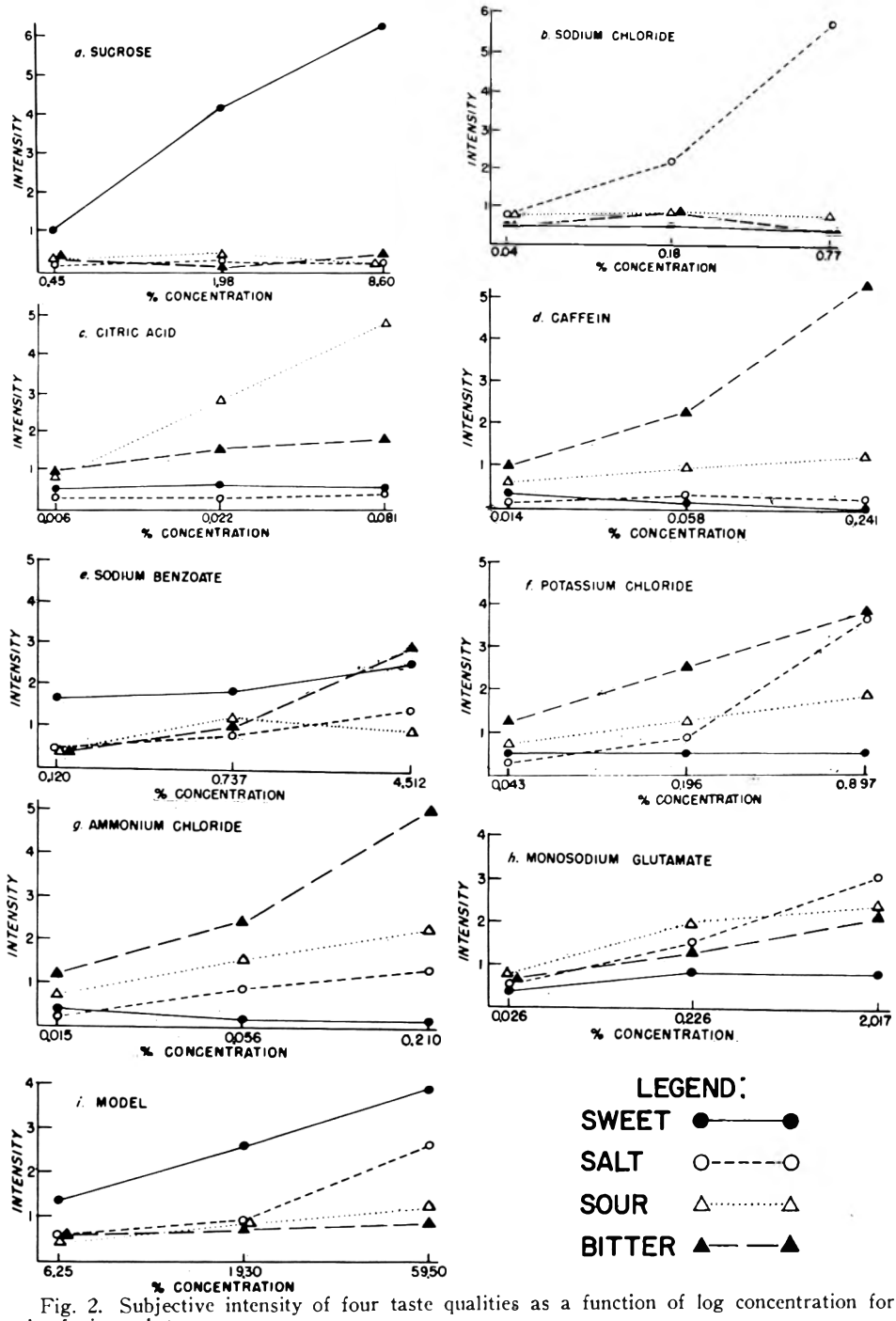

Fig. 2. Subjective intensity of four taste qualities as a function of log concentration for each of nine substances.

Table 2. Replicate error distributed according to the four independent variables.

		Subjects [a]	
		Range	Number in range
Substance:			
Water	0.6		
Sucrose	0.8		
Sodium chloride	1.1	2.8–3.2	1
Caffein	1.3	2.3–2.7	3
Citric acid	1.4	1.8–2.2	4
Sodium benzoate	1.7	1.3–1.7	6
"Model" solution	1.7	0.8–1.2	5
Ammonium chloride	1.8	0.3–0.7	5
Potassium chloride	1.9		
Monosodium glutamate	2.0		

Quality: [b]		Concentration: [b]	
Sweet	1.1	Low	0.9
Salt	1.4	Medium	1.4
Sour	1.8	High	2.2
Bitter	1.9		

[a] Results for water included.
[b] Results for water not included.

all four qualities rated about the same when data were averaged across the entire experiment.

Table 3 presents information obtained in the analyses of variances done separately for each substance. Average variances for selected effects are shown. Most effects were highly significant, and it is the relative magnitudes that are of major interest.

Subject effect was small but significant with all substances, including water. This was expected, and is simply a demonstration that people have different ways of using scales. Again as expected, there was an important effect due to concentration (fortunately, it did not appear with water). By far the largest variances arose with quality. This demonstrates analytically what the charts show, namely, that there were reliable differences among the qualities used to describe each substance. Note the large variance for sucrose, where one quality was clearly dominant, and the relatively small variances for sodium benzoate and monosodium glutamate, where all four qualities were used to an important extent.

The quality-concentration interactions vary considerably, but have no major importance for our present purpose. This interaction arose from the fact that, as concentration changed, the relative importance of the qualities changed. In one sense, this is an artifact, since, at the "low" concentration, all qualities were of necessity near the zero level.

Subject-quality interaction has a particular significance with reference to the problem under investigation. It is a measure of the extent to which the subjects differed in the single qualities, or patterns of qualities, which they used to describe the substances. It shows that the multiple qualities, as displayed in the charts, occurred because people differed in the qualities used, and not because everybody used the same multiple qualities. A significant subject-quality interaction was found for every substance but water. It varied from a low of 1.7, for sucrose, to a high of 8.9, for monosodium glutamate. It is generally lower, indicating more agreement among subjects for the four familiar substances (the "pure" tastes) except for citric acid, which is exceeded only by sodium benzoate and monosodium glutamate.

The data so far presented have demonstrated the generality of the sodium benzoate effect. Different people responded in different ways to the other substances as well. Sodium benzoate is not unique; even such common things as sucrose

Table 3. Average variance attributable to various sources in the analyses of variance for ten substances.

Substance	Subject 23 [a]	Quality 3 [a]	Concentration 2 [a]	Quality-concentration 6 [a]	Subject-quality 69 [a]	Replicate error 288 [a]
Water	2.9	2.9	0.1 [b]	1.0 [b]	0.8 [b]	0.6
Sucrose	3.4	438.7	89.4	83.1	1.7	0.8
Sodium chloride	5.2	188.8	67.0	83.0	3.2	1.1
Citric acid	5.3	182.0	78.3	41.3	6.4	1.4
Caffein	4.6	229.4	66.6	57.3	2.8	1.3
Sodium benzoate	7.2	39.8	87.5	11.4	6.5	1.7
Potassium chloride	5.3	107.1	155.4	31.9	6.3	1.9
Ammonium chloride	8.5	182.2	116.1	36.5	4.8	1.8
Monosodium glutamate	5.9	31.1	106.7	11.6	8.9	2.0
"Model" solution	6.3	119.5	90.4	16.0	3.7	1.7

[a] Degrees of freedom.
[b] Except for these, all effects were significant at or below the 1% level.

and sodium chloride show some of the effect, and monosodium glutamate shows it to an even greater extent than sodium benzoate.

Do the results support the hypothesis that familiar substances will show less of such variation than the relatively unfamiliar? The two most appropriate indices of variability in this experiment were replicate error, a measure of intra-individual variation, and subject-quality interaction, a measure of differences among people in the qualities used. Table 4 ranks the familiar and unfamiliar substances from low to high on these two indices. On subject-quality interaction there is only one departure (citric acid) from the rule that familiar substances show less variability. On replicate error the data are in perfect agreement: the four familiar substances occupy the first four ranks. Thus the results tend to support Hypothesis 2, but with some qualification.

Table 4. Comparison of variability of response to familiar and unfamiliar substances.

	Subject-quality interaction rank	Replicate error rank [a]
Familiar substances:		
Sucrose	1	1
Sodium chloride	3	2
Citric acid	7	4
Caffein	2	3
Unfamiliar substances:		
"Model" solution	4	5.5
Monosodium glutamate	9	9
Potassium chloride	6	8
Ammonium chloride	5	7
Sodium benzoate	8	5.5

[a] Ranked from low to high.

The outcome on Hypothesis 3 is not so clear-cut. It is assumed that learning, along with physiology, plays an important role in determining how people respond to taste stimuli, and that a person tends to develop constant tendencies to respond in certain ways so that he is more likely to use some qualities than others. In other words, he has "preferred" and "non-preferred" qualities. With familiar substances, people have learned common ways of responding. Hence, personal idiosyncracies should be less important; however, with unfamiliar substances, these individual tendencies should appear in greater strength.

An analysis was made to test this hypothesis. Each subject's average rating for each quality, across all concentration levels of the nine substances (excluding water), was obtained, then "corrected" by subtracting the subjects' own over-all average (to adjust for his possible tendency to rate high or low) and the over-all average level for the quality (to adjust for the fact that the substances, as a group, were actually stronger in some qualities than others). Comparisons of the residuals permitted identification of "preferred" qualities (those used more often or rated higher) and "non-preferred" qualities (those used less often or rated lower). Most subjects showed such tendencies: 14 had one "preferred" quality, 15 had one "non-preferred" quality, 11 had both.

The pattern of qualities used by each subject to describe each substance was determined by inspection. Table 5 displays the patterns for sucrose and caffein, two of the least variable substances, and for sodium benzoate and monosodium glutamate, the two most variable substances. With the familiar substances, the patterns were regular and could have been predicted from the average curves. Most people used the "appropriate" quality although they might vary in the "other"

Table 5. Combinations of taste qualities used by test subjects to describe each of four substances.

Substance	Combination	No. of subjects using
Sucrose:	Sweet	19
	Sweet-sour	2
	Other	3
Sodium chloride:	Salt	11
	Salt-sour-bitter	3
	Salt-sweet-sour	4
	Salt-bitter	3
	Salt-sour	2
	Salt-sweet	1
Sodium benzoate:	Bitter-sweet	6
	Sweet-sour	3
	Sweet-salt-sour-bitter	3
	Bitter	2
	Sweet	2
	Sweet-salt-bitter	2
	Other	6
Monosodium glutamate:	Salt-sour-bitter	5
	Sweet-salt-sour-bitter	6
	Sour	3
	Bitter	3
	Salt-sour	2
	Other	5
Citric acid:	Sour	5
	Bitter	2
	Sour-bitter	10
	Sour-salt	3
	Sour-sweet	2
	Other	2

qualities mentioned. It was clear that the qualities, and patterns of qualities, used here were determined primarily by the substance. But for the five unfamiliar substances the patterns were many and various, like the two examples shown.

Individual patterns on the five unfamiliar substances were examined to determine whether they were consistent with the subjects' "preferred" and "non-preferred" qualities. Of 120 cases (24 *S*'s × 5 substances) the consistent cases outnumbered the inconsistent cases about 2 to 1; however, in about one-third of the cases no determination could be made. It was concluded that constant tendencies, as here defined, are not very good predictors of responses to particular unfamiliar substances; although there is some relationship.

Further evidence on this point was sought by a correlation method. If most people respond to familiar substances in the same way because the responses are determined by the nature of the substance and the function of the taste receptors, ratings should not be correlated between substances; on the other hand, if responses to unfamiliar substances are determined to a large extent by personal factors, not necessarily related to the substance or taste receptor function, these ratings should tend to correlate positively.

The complete matrix of inter-substance correlations was derived for each quality. Significant positive correlations occurred about twice as often among the unfamiliar substances as among the familiar substances, which would tend to support the hypothesis; however, familiar substances were positively correlated with the unfamiliar substances about as often, something that would not be predicted by the hypothesis. Thus, the analysis provided only weak support for the "constant tendencies" hypothesis.

CONCLUSIONS

The generality of the sodium benzoate effect was proven. For people to use different qualities to describe the same substance appears to be the rule rather than the exception. Familiar substances tend to show less of this variability than do substances that represent new taste experiences. Although individual constant tendencies to respond with certain qualities could be identified, they did not appear to be important determiners of the patterns of response even to unfamiliar substances. The hypothesis that learning is an important cause of interpersonal variability was weakly supported, but not proven.

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Nutrient Content of Morel Mushroom Mycelium: Amino Acid Composition of the Protein

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SUMMARY

Mycelia of three species of morel mushrooms, *Morchella crassipes*, *M. esculenta*, and *M. hortensis*, were grown in submerged culture in a glucose-ammonium phosphate-corn steep liquor medium. The dried mycelia were subjected to proximate analyses and amino acid determinations. A commercial morel mushroom powder was also analyzed for the same constituents. The dry samples contained 22.8–51.0% protein and 2.18–7.55% fat, depending upon the species. Amino acid contents were similar to those reported in the literature for other fungi.

INTRODUCTION

Previous studies have shown that morel mushroom mycelium can be grown in submerged culture with glucose, maltose, lactose, or wastes containing these sugars as substrates (Litchfield *et al.*, 1963; Litchfield and Overbeck, 1963).

Robinson and Davidson (1959) and Block (1960) pointed out the potential value of mushroom mycelium as a source of protein for man or domestic animals. Block *et al.* (1953) reported the qualitative amino acid composition of the mycelium of the Basidiomycete *Agarius blazei*, grown in submerged culture, and Reusser *et al.* (1958) reported quantitative data on the essential amino acid composition of *Tricholoma nudum*. However, as far as it can be determined, there have been no detailed reports of the composition of morel mushroom mycelium, especially the amino acid composition of the proteins of these organisms.

This paper presents both proximate analyses and amino acid analyses of the mycelia of three species of morel mushrooms, *Morchella hortensis*, *M. esculenta*, and *M. crassipes*.

EXPERIMENTAL METHODS

Microbiological methods. The cultures of *M. hortensis*, *M. esculenta*, and *M. crassipes* were those used in previous studies (Litchfield *et al.*, 1963; Litchfield and Overbeck, 1963) and were maintained in the same manner. All three organisms were grown in the submerged-culture vessels described by Litchfield *et al.* (1963). The medium used contained the following constituents: 2.5% glucose monohydrate (Cerelose; Corn Products Company, Argo, Illinois); 0.2% $(NH_4)_2HPO_4$; 1% corn steep liquor (Corn Products); and 0.2% $CaCO_3$. Antifoam (Dow Corning AF) was added to the medium initially at a level of 0.05% to prevent excessive foaming during the growth cycle. The media, in culture vessels, were inoculated and aerated at 25°C—5 days for *M. hortensis* and *M. esculenta*, and 7 days for *M. crassipes*.

The level of aeration used was 0.25 L of air per liter of medium per minute, which corresponded to an oxygen absorption rate of 0.08 millimole of oxygen per liter per minute as determined by the sulfite oxidation method of Cooper *et al.* (1944) as modified by Corman *et al.* (1957). The mycelia were harvested and dried according to procedures described previously (Litchfield *et al.*, 1963).

Chemical methods. Protein, fat, moisture, and ash contents of the dried mycelia were determined according to methods of the AOAC (1960).

Acid hydrolysates for amino acid determinations were prepared by autoclaving approximately 100-mg samples with 6*N* HCl in sealed tubes for 6 hr at 121°C. Alkaline hydrolysates were prepared in a similar manner using 5*N* NaOH. Amino acids, except for tryptophan, were determined with the Technicon Auto Analyzer according to Piez and Morris' (1960) modification of the automatic gradient elution ion-exchange chromatographic procedure of Spackman *et al.* (1958).

Tryptophan was determined in alkaline hydrolysates by spectrophotometric procedures described by Spies and Chambers (1948) and Spies (1950).

RESULTS AND DISCUSSION

Proximate analyses. Table 1 shows proximate analyses of the mycelia of three species

Table 1. Proximate analyses of dried morel mushroom mycelia.

Organism	Composition [a] (g/100 g dry weight)				
	Dry matter	Nitrogen	Protein	Fat	Ash
M. crassipes	95.3	3.65	22.8	7.55	18.2
M. esculenta	96.2	4.00	25.0	3.31	17.3
M. hortensis	97.7	4.31	26.9	3.13	17.7
Morel Mushroom Flavoring	96.1	8.17	51.0	2.18	6.44

[a] Average of duplicate analyses.

of morel mushrooms and a commercial product labeled "Morel Mushroom Flavoring" (Special Products, Inc., Springfield, Missouri). It was pointed out previously that the composition of morel mycelium depends on the composition of the medium in which it is grown (Litchfield *et al.*, 1963).

The dried mycelium of *Morchella crassipes* consistently showed a greater fat content than either *M. esculenta* or *M. hortensis* grown in the same medium and under similar conditions, and is probably characteristic of this species. Protein contents of the mycelium vary somewhat with the nitrogen content of the corn steep liquor used in the growth medium.

The protein content of the commercial product was considerably higher than the values obtained in previous studies. A maximum of 38% protein was obtained in *M. hortensis* after growth in a nitrogen-rich medium (Litchfield *et al.*, 1963). Similar results have been reported by other investigators for other fungi.

The high ash values indicate the great extent of assimilation of mineral constituents from the growth medium. The calcium carbonate used for neutralizing the medium during the growth phase also contributed to these values. However, the majority of mineral constituents in the mycelium originated from the corn steep liquor component of the medium, which analyzed 20% ash.

Amino acid composition. Table 2 shows the amino acid compositions of the various morel mushroom products. *M. esculenta* protein contained approximately twice the amount of arginine present in *M. hortensis*

Table 2. Amino acid composition of morel mushroom mycelia.

Amino acid	Composition (g per 16 g protein nitrogen)				
	M. hortensis	*M. crassipes*	*M. esculenta*	Morel mushroom flavoring	*Tricholoma nudum* (Reusser et al., 1958)
Alanine	4.45	6.16	4.81	11.12	
Arginine	3.96	3.00	7.95	4.32	4.63
Aspartic acid	4.63	4.82	4.97	6.02	
Cystine	0.36	0.42	0.27	0.80	
Glutamic acid	15.43	14.10	14.81	13.45	
Glycine	3.01	3.12	2.94	2.75	
Histidine	1.88	1.98	2.12	1.94	2.96
Isoleucine	2.40	2.87	2.70	3.45	3.15
Leucine	5.03	5.57	5.12	6.06	6.66
Lysine	3.02	3.46	3.84	5.38	6.66
Methionine	0.69	1.01	0.90	1.41	1.66
Phenylalanine	2.28	1.90	2.51	2.82	3.70
Proline	4.52	5.11	4.15	4.78	
Serine	2.82	3.08	3.05	3.90	
Threonine	2.68	2.99	2.98	3.37	3.70
Tryptophan	0.98	1.48	0.86	1.43	3.52
Tyrosine	1.85	1.70	1.72	2.49	
Valine	2.94	3.04	3.36	3.86	3.89

or *M. crassipes* mycelium or in "Morel Mushroom Flavoring." The proteins of *M. crassipes* and the commercial morel product contained significantly greater quantities of alanine than did the mycelial protein of the other organisms. In general, the contents of all the other amino acids in the proteins of the four samples were quite similar.

In addition to the amino acids listed in Table 2, trace amounts of cysteic acid, ethanolamine, methionine sulfoxide, methionine sulfoxamine, and homocitrulline were detected in *M. esculenta*, *M. hortensis*, and Morel Mushroom Flavoring, but not in *M. crassipes*.

The mycelia of *M. crassipes*, *M. esculenta*, and *M. hortensis* respectively contained 1.15, 2.00, and 2.00% (dry-weight basis) of α-aminoisobutyric acid, whereas the "Morel Mushroom Flavoring" did not contain this compound in detectable concentration. *M. crassipes* mycelium did not contain α-amino-*n*-butyric acid, but *M. esculenta* mycelium contained 0.25%. The other samples showed trace amounts. All of the samples contained trace amounts of 2,4-diaminobutyric acid.

It is interesting to compare these data with those reported for *Agaricus blazei* by Block *et al.* (1953) and for *Tricholoma nudum* by Reusser *et al.* (1958). *A. blazei* protein contained all of the essential amino acids for man, but no quantitative values were presented. The amino acid composition of *T. nudum* is shown in Table 2 for comparison. These values are quite similar to those obtained for the morel mycelia except for the lysine, phenylalanine, and tryptophan contents, which are somewhat higher in *T. nudum*.

In general, however, the amino acid compositions of the morel mycelia are quite similar to values reported for fungi (Anderson and Jackson, 1958). It appears that these organisms have primary value as flavor supplements in foods and have the incidental value of contributing certain amino acids.

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