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Changes in Water-Soluble Calcium and Magnesium Content of Pear Fruit Tissue During Maturation and Ripening in Relation to Changes in Pectic Substances

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SUMMARY

Changes were determined in total and water-soluble calcium and magnesium content and in the pectic substances of Bartlett pears during maturation and storage. The data indicate a change in metabolic processes when the fruit is removed from the tree. In maturing fruit, about 48% of calcium and 65% of magnesium are present in soluble form. Both cations generally decreased during maturation. The Mg/Ca ratio varied with growing conditions and may be related to soil composition. The data indicate that marc, total pectin, water-soluble pectin, total calcium, and total magnesium are correlated with firmness. The relationship of soluble calcium and magnesium to firmness is radically different in detached fruit from that in fruit on the tree. Analyses of data indicate that the "bound" calcium and magnesium are present in concentrations far greater than the total available carboxyl groups of pectin, and that protopecin content is not related to calcium- or magnesium-bound pectinic acid chains. In detaching ripening fruit, when a general breakdown begins there is a good correlation of all data with firmness.

INTRODUCTION

The softening of deciduous fruit tissue during ripening on the tree is accompanied by an increase in water-soluble pectic substances, a decrease in protopectin content, originally reported by Frémy (1848), and a decrease in degree of esterification and degree of polymerization of extracted pectins (Kertész, 1951; Hulme, 1958). The older data reported, however, are incomplete even for the more widely investigated apple fruit, and indicate trends rather than close quantitative correlations. Typical of such trends is that of McCready and McComb (1954), who reported that extractable pectin increased from 39% in unripe pears to 60% in ripe fruit, that degree of esterification decreased from 89% to 43%, and that intrinsic viscosity of extracted pectins decreased from 6.0 to 1.5. Complete quantitative data on the transformation of protopectin into pectins, however, are limited even for apples, and only a few detailed investigations with pears have been reported (Weurman, 1952). Date and Hansen (1954) investigated changes occurring in pectic constituents of three varieties of pears during cold storage and reported ripening to be accompanied by increase in both protopectin and total pectin. Increase in total pectin content in pears during ripening was also reported by Yagubov (1958), who reported an initial increase followed by decrease as pears ripened for six varieties investigated. Increases in soluble pectin content and decreases in protopectin content fluctuate very frequently, and these fluctuations are usually greater than can be accounted for by experimental error (Hulme, 1958). The insolubility of protopectin is believed to be due to the presence, particularly in the middle lamella,

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of pectinic acid micelles, ionically bound by cations such as Ca^{++}, Mg^{++}, or Fe^{+++}. In early investigations, both calcium and magnesium were observed in plant tissue and in the pectins extracted; later, only calcium pectinates were believed to occur. Published data are considerable on the calcium content of fruit tissue, and limited on the occurrence of other cations associated with pectinic acids (Hulme, 1958). There is only one known reference, however (Jacquin, 1958), indicating that the ratio of Ca/Mg may be affected by the composition of soil.

To obtain more information on the factors influencing the firmness of pear fruit, investigation was made of changes in the pectic substances during maturation on the tree and in storage, and of ripening in relation to the content of total and water-soluble calcium and magnesium. The data are reported herein and are discussed in relation to ripening and to decrease in firmness.

EXPERIMENTAL

Preparation and analysis of pear tissue. Two sets of Bartlett pears were prepared: freshly harvested pears, to determine changes during growth; and detached fruit held in cold storage and subsequently ripened, to determine postharvest changes during storage.

Two hundred and fifty pears were labeled in early June with numbered tags. On each weekly harvest date, 25 fruits were harvested in numerical sequence. For example, on the first date, samples marked 1, 11, 21, 31, etc., were harvested. On the second date, 2, 12, 22, 32, etc., were harvested. In all, 10 harvests were made. The harvested pears were arranged on the table in numerical order, and the largest diameter of each fruit was measured. While the fruits were lined up, every other pear was removed to obtain a representative sample for pectin determination. The remaining pears were used for further chemical analysis. In addition to diameter and weight, flesh firmness of fruit tissue was determined with a Magness-Taylor type of pressure tester. The fruit used in storage investigations was held at 41°F for various periods and then ripened at 68°F. The cold-storage experiments also included long-term storage at 41 and 32°F.

Analyses were made for the following constituents: soluble solids, total solids, total and soluble calcium and magnesium, tannin, alcohol-insoluble solids, total and soluble pectin, methoxyl, acetyl, and total esterified pectin.

Calcium and magnesium content. The fruit used for calcium, magnesium, potassium, and phosphorus analyses were gently scrubbed in tap water, rinsed with distilled water, and wiped dry. For determination of calcium and magnesium, a 200-g sample was comminuted for 5 min in an electric blender, and the slurry was transferred to a 500-ml volumetric flask. The air was removed by gently rotating the contents of the flask while under reduced pressure. Since the specific gravity of the slurry was close to that of water, differing by less than 0.5%, aliquots were withdrawn on a volume basis. For the analysis of the slurry, 200 ml were withdrawn during continuous agitation of the contents of the flask. The remainder of the slurry was centrifuged and the supernatant portion filtered through No. 1 Whatman paper. Both the slurry and the filtrate were evaporated and ashed in Vitreosil dishes (Esau, 1961). The ash was analyzed for calcium, magnesium, and potassium by the modified flame-photometer method described by Brown *et al.* (1952). Portions of the above fruit in sliced form were also ashed and analyzed similarly.

Tannin content. The tannin-like material was determined on a sample of fruit flesh obtained from peeled and cored fruit prepared and analyzed by the procedure of Leonard *et al.* (1954).

Preparation of marc (alcohol-insoluble residue). The alcohol-insoluble solids from the pears were prepared by a modification of the procedure of Postlmayr *et al.* (1956) and Gee *et al.* (1958). After quartering and removal of the core, the blossom end, and the stem fibers, 500 g of fresh pear tissue were weighed out and, without delay, thinly sliced with a stainless-steel knife into 650 ml of boiling acetone to give a final concentration of about 60% acetone. Boiling was continued for 30 min under reflux. The contents of the flask were then transferred to a glass jar and stored at 32°F until further use.

The acetone was discarded before further processing, and the sliced fruit was comminuted for 1–2 min to 70% ethanol in an electric blender. After filtering through No. 1 Whatman paper on Buchner funnel, the pulp was washed twice with 70% ethanol. The pulp was filtered drip dry and then transferred to a beaker, covered with a mixture of 700 ml of 95% ethanol, 250 ml water, and 50 ml HCl, and stirred with a mechanical stirrer for 1½ hr. After the acid treatment, the slurry was filtered through a fresh filter paper and washed on the funnel with 70% ethanol until free of chlorides. The chloride-free pulp was covered with acetone, stirred in the funnel, and set aside to drip, and this procedure was repeated once more. The pulp was then transferred to a beaker, covered with acetone, and set aside overnight to

remove residual alcohol and salts. Next day, the acetone was filtered off and the pulp dried at 37°C until the following morning to remove organic solvents.

The final product was almost paper-white, its ash content was about 0.15%, and the total cation content amounted to 18–20% by weight of ash. The dried marc was weighed and set aside for 24 hr to pick up atmospheric moisture to facilitate grinding in a mill, such as Wiley, to pass a 40-mesh screen. Some very fluffy material did not pass through the screen. To avoid fractionation of the marc, the material remaining in the mill was added to the ground sample and mixed well.

Analysis of pectic constituents in the marc. The total pectin content was determined by the Versene-pectinase procedure of McCready and McComb (1952). A 0.5-g sample of marc, dampened with 1–2 ml of purified ethanol, to facilitate dispersion, was mixed with 150 ml of 0.5% solution of reagent-grade disodium Versenate, adjusted to pH 11.5 with 2*N* NaOH and stirred on a magnetic stirrer for 2 hr. The pH was then adjusted to 5.0 with 50% acetic acid, 2 ml of 10% solution of Pectinol R-10 was added, and the mixture stirred again for 2 hr. The mixture was then transferred to a 250-ml volumetric flask, made up to volume, and filtered, and 10 ml of the filtrate was diluted to 100 ml. Two ml of this dilution were used for the carbazole procedure. The protopectin content of the marc was determined by first removing water-soluble constituents by four successive extractions with water. A 0.5-g sample of the marc, dampened with 1 ml ethanol, was weighed into a 250-ml flat-bottom centrifuge cup, covered with 100 ml water, and stirred on a magnetic stirrer for 2 hr. The mixture was centrifuged after de-aeration under vacuum for 1–2 min to facilitate sedimentation. The water extract was then siphoned off and discarded. The residue was extracted three more times, at 2-hr intervals. After the last extraction, two drops of toluene and 100 ml of water were added to the residue, mixed, and stored overnight. Next day the sediment was stirred up and centrifuged, the water extract discarded, and the residue, from this point, treated as the sample for total pectin determination, omitting the 1–2 ml alcohol. After addition of Pectinol, the mixture was then transferred to a 250-ml volumetric flask, with 2 drops of toluene added, stored overnight, filtered next day, made up to volume, and analyzed, as for total pectin.

The Versene-soluble pectic substances were determined by first extracting with water 0.5-g portion of the marc as described under protopectin; then the centrifuged residue was transferred with 100 ml of 0.5% Versene solution into a 250-ml beaker, the mixture adjusted to pH 6.0, and stirred for 2 hr. It was then centrifuged and the residue covered with 100 ml water. This was repeated two more times, at 2-hr intervals. After the last extraction, the residue was covered with 100 ml water, mixed, and stored overnight with toluene. Next day the contents of the centrifuge cup were washed into a 500-ml volumetric flask containing the previous Versene extracts and subsequent wash, made to volume, and filtered. A 50-ml portion of filtrate was then adjusted to pH 11.5, set aside for 2 hr, and adjusted to pH 5.0, 2 ml of 10% Pectinol R-10 added, and the mixture was again set aside for 2 hr. The extract treated with Versene and pectinase was then transferred to a 250-ml flask and diluted to volume, and 2 ml was used for carbazole assay.

For the characterization of extracted pectic substances, a 1-g portion of the marc was weighed into a 250-ml centrifuge cup, dampened with 1–2 ml ethanol, covered with 100 ml water, mixed, de-aerated, and stored overnight with added toluene as a preservative. Next day the mixture was stirred 3 hr and centrifuged. The supernatant liquid was siphoned off and passed through 10 ml of Amberlite 120 resin in the H form to remove the cations that may have been extracted by water in the form of salts. The extraction was repeated twice, at 2-hr intervals, with 75 ml water, and these extracts also passed through the column, collected in a 250-ml volumetric flask, and stored overnight with toluene. After the last extraction, 75 ml of water were added to the centrifuge cup and stored overnight with toluene. Next day the final water extract was separated by centrifuging and also passed through the resin column. The total pectin content of the composite water extracts was determined by saponifying 25 ml of extract for 2 hr with 5 ml of 0.5*N* NaOH. The solution was then adjusted to pH 5.0, treated 2 hr with 2 ml of 10% Pectinol R-10, transferred to a 500-ml volumetric flask, and made up to volume, and 2 ml was used for the carbazole assay.

Each 2-ml portion used for carbazole assay of total pectin, protopectin, and Versene-soluble pectin represented 0.4 mg of marc as given under preparation of marc.

The water-soluble pectin was characterized by the titrimetric method of Anyas-Weisz *et al.* (1951). The free carboxyl groups were determined by titrating a 50-ml portion of the cation-free extract with 0.1*N* NaOH. The total ester groups were determined by adding 5.0 ml of 0.5*N* NaOH to this solution, setting it aside for 1 hr, and then back-titrating with 0.25*N* HCl. The total carboxyl groups were determined by saponifying a 25-ml portion for 1 hr with 5.0 ml

of 0.5*N* NaOH, percolating through 10 ml of washed Amberlite MB 1 in a 12-mm column to remove low-molecular acids, washing the column twice with water, and titrating the percolate with 0.1*N* NaOH. The acetyl content was determined as the difference between the total carboxyl content and the total uronic acid carboxyl content.

Some of the marc samples were also titrated directly by the modification of the Hinton titration procedure described by Owens *et al.* (1952).

Expression of results. The results are expressed on a fresh-fruit basis, though this is unsatisfactory because of changes in weight of the fruit during growth and during storage. The increase in size observed under our conditions was due not to increase in numbers of cells but to increase in size per cell, through uptake of moisture, sugars, and other carbon compounds. This is reflected in the fact that the total solids remain essentially constant during this period, as shown in Table 1, and that the weight of alcohol-insoluble solids content shown in Fig. 1 per average fruit does not change after the first stage of growth, since the cell-proliferating stage has now been passed. Jermyn and Isherwood (1956) found, for Kieffer and Conference pears, that cell wall material changed little after a certain early stage of growth, and that the weight increase in fruit was mainly due to an uptake of water and sugars. The mean weight of cell wall material per pear was much the same for pears weighing 60 and 160 g. Our limited, similar, data are shown in Fig. 1, wherein marc content is plotted as found and as percent of fruit tissue against weight of average pear and against harvest dates, wherein time of harvest is the independent variable.

Weight changes from loss of moisture and the respiration of reserve carbohydrates in storage give constituents such as total cations the appearance of changing significantly although they actually do not change. The apparent changes are reflected in changes in cation content and pectin content, as usually reported in the literature. Jermyn and Isherwood (1956) found considerable loss in weight during cold storage of Conference pears from the firm-green stage to the yellow-slushy stage, from 4.4–4.5% at 7 days to 10.0–11.2% at 31 days. Nevertheless, total polysaccharide content expressed on a per-pear basis decreased up to 11 days of storage, then fluctuated, and finally increased in later stages of storage. The total polysaccharide content changed from 3.56 g per pear at time of harvest to a minimum of 3.00 g at 23 days, and then increased to 3.40 g at 31 days. When calculated on a weight basis, the data of

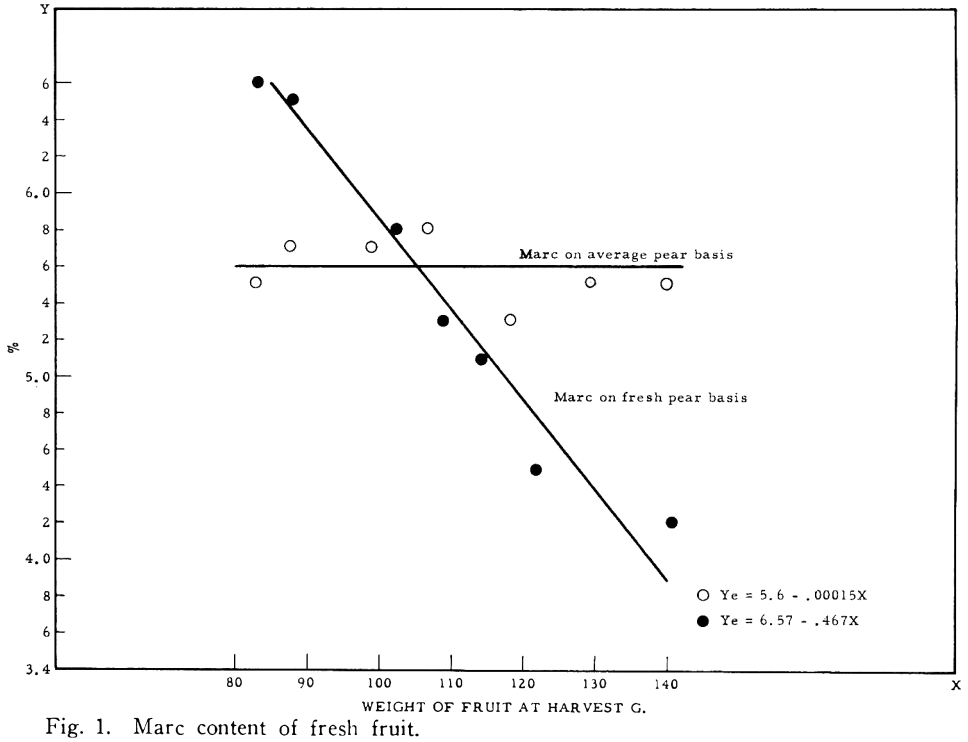

Fig. 1. Marc content of fresh fruit.

Jermyn and Isherwood (1956) indicate that polysaccharide content per 100 g of pear would vary from 2.23 to 1.93 to 2.15 g.

In the fruit used in the present investigation, excessive loss of moisture was prevented by using a commercial packing method, wrapping in paper, and storing in closed cartons. Weight loss during 30 days of storage at 32°F was 1.6%, and an additional 1.6% during ripening in a humidified atmosphere at 68°F. Storage at 41°F caused somewhat greater total loss—4.8% in 45 days.

RESULTS AND DISCUSSION

Soluble and total calcium and magnesium content. Table 1 shows changes in Ca, Mg, K, P, and tannins in relation to changes in diameter, weight, firmness, and total and soluble solids content during maturation of pears on the tree. Table 2 shows the content of Ca and Mg "as found" and also corrected for the gain in weight of the fruit.

Calcium is mentioned in the literature as the principal cation in pears, although some data on magnesium have been reported (Hulme, 1958). The data in Table 1 on Mg/Ca ratios are the reverse of those reported by Jacquin (1958). The principal cation at the tree root level in Davis soil, a Yolo loam of pH 6.5–7.3, is magnesium, and the Mg/Ca ratio for pears grown at Davis is 2.2:1. In fruit grown in the Sierra Nevada foothills (Placerville) in acid soils of pH 5.5, this ratio drops to 1.1:1, as shown in Table 4. The average total Ca content (Table 1) was 45 ppm, and magnesium was 93 ppm. Both cations fluctuate considerably during the maturation period. Doesburg

Table 1. Changes in sizes, firmness, and composition of pears during maturation.

	Harvest date									
	6-30	7-7	7-14	7-21	7-28	8-4	8-12	8-18	8-25	9-1
Diam. increase (%)[a]	0.0	3.4	6.6	10.0	13.3	16.6	20.0	23.4	26.6	30.0
Wt. increase (%)[a]	0.0	7.5	15.0	23.0	31.0	38.5	46.0	54.5	62.5	70.0
Firmness (lb)	23.3	21.8	21.2	20.5	23.5	19.1	17.6	16.0	14.6	13.2
Soluble solids (%)	10.9	11.0	11.8	10.8	11.5	12.8	12.0	12.8	12.8	12.8
Total solids (%)	15.1	15.2	15.4	15.7	15.7	15.1	15.2	.....	.....	15.4
Ash (%)	.370	.....	.390	.....	.350	.289	.286	.280	.....	.255
pH	4.3	4.5	4.5	4.7	4.5	4.5	4.5	4.5	4.3	4.2
ml 0.1*N* alk. to pH 7.0 per 10 g	4.2	3.6	3.4	3.6	4.0	2.6	4.6	3.2	3.6	1.6
Tannins mg/100g	55.7	61.0	66.7	60.8	55.3	51.8	52.8	57.5	57.6	63.8
Fruit as is (ppm)										
Ca	62	42	39	44	47	52	36	40	40	48
Mg	140	89	87	106	112	114	87	89	78	90
P	127	129	130	117	111	104	120	140	98	96
K	1255	1175	1280	1135	1320	1370	1225	1320	1160	1225
Whole slurry (ppm)										
Ca	46	45	41	47	49	51	38	44	39	46
Mg	100	95	91	109	113	111	95	86	73	81
P	129	134	139	111	104	106	122	113	95	97
K	1288	1176	1370	1200	1132	1356	1355	1275	1150	1125
Slurry filtrate (ppm)										
Ca	20	22	20	17	25	29	18	21	16	25
Mg	66	66	60	73	63	76	59	59	43	58
P	108	80	127	102	93	100	117	103	80	96
K	1150	1043	1215	1135	1030	1270	1235	1180	915	1120
Ratio Mg/Ca										
Fruit as is	2.3	2.1	2.3	2.4	2.4	2.2	2.4	2.2	2.0	1.9
Whole slurry	2.1	2.1	2.2	2.3	2.3	2.2	2.5	1.9	1.9	1.8
Slurry filtrate	3.3	2.9	3.0	4.3	2.5	2.6	3.3	2.8	2.7	2.3

[a] Percent increase from regression graphs.

Table 2. Regression corrections of various forms of Ca and Mg (ppm).

	Harvest date									
	6-30	7-1	7-14	7-21	7-28	8-4	8-12	8-18	8-25	9-1
Wt. correction from regression lines (%)	0.0	7.5	15.0	23.0	31.0	38.5	46.0	54.5	62.5	70.0
Total Ca										
Found (ppm)	46	45	41	47	47	51	38	44	39	46
Regress. Value	52.8	52.0	51.0	50.8	50.0	49.2	48.8	48.0	47.2	46.8
Wt. corrected per fruit	52.8	55.9	58.6	62.5	65.5	68.2	71.8	74.2	76.8	79.6
Soluble Ca										
Found (ppm)	20	22	20	17	25	29	18	21	16	25
Regress. value	24.5	24.5	24.0	24.0	24.0	24.0	23.5	23.5	23.5	23.5
Wt. corrected per fruit	24.5	26.3	27.6	29.5	31.4	33.2	34.2	36.2	38.2	39.9
Total Mg										
Found (ppm)	100	95	91	109	11.3	111	95	86	73	81
Regress. value	106.0	103.6	101.2	99.0	96.4	94.0	91.6	89.2	87.0	84.4
Wt. corrected per fruit	106.0	111.4	117.7	121.8	126.3	130.2	133.8	137.8	141.5	143.4
Soluble Mg										
Found (ppm)	66	66	60	73	63	76	59	59	43	58
Regress. value	69.5	68.0	66.0	65.0	63.0	61.5	60.0	58.0	57.0	55.0
Wt. corrected per fruit	69.5	73.1	75.9	79.9	82.5	85.2	87.6	89.6	92.6	93.5
From regression values										
Soluble Ca (%)	46.5	47.2	47.0	47.2	48.1	48.7	48.1	48.9	49.8	50.2
Soluble Mg (%)	65.6	65.4	65.3	65.6	65.6	65.4	65.2	64.9	65.5	65.5

(1957) also observed fluctuation in the calcium content of pears. Our data suggest that the rise might coincide with orchard irrigation.

The calcium and magnesium contents of the slurry filtrate representing soluble calcium and magnesium salts, are low and show no trend with maturation. From the regression values in Table 2, the percentage of soluble calcium is 46.5 of total calcium at the first picking, and 50.2 at the last picking. The corresponding values for magnesium are 65.6 and 65.5%. There is no apparent increase in soluble calcium and magnesium content with advancing maturation, unlike with apples as observed by Doesburg (1957). His data indicate that soluble calcium varied from 46 to 54% of the total calcium in green apples, and 42–52% of the total in ripe apples. In storage, his data indicate, the soluble calcium decreased to 34–40% of the total, although soluble pectin content continued to increase. The pH of the cut surface of apple tissue decreased on standing, and reached an equilibrium value. The pH of the cell juice was equal to that of the surface just after cutting. An exchange of cations (particularly K^+ and H^+) occurred between the cations of the cell wall and calcium bound by pectic substance during the period of solubilization of pectin. We have observed that the pH of the juice expressed from pear tissue was higher than that of purée prepared by blending the tissue. If cation exchange occurred as postulated by Doesburg, the pH of cell walls would decrease during the ripening period, when solubilization of pectin was rapid. He reported that the pH of apple juice increased during ripening. Weurman (1952) reported a lowering of pH with the maturation of pears.

The correction for changes in weight of fruit was calculated from the regression data, and this correction was then applied to total and soluble calcium and magnesium contents, as shown in Table 2. Calcium apparently accumulates in fruit during maturation, and magnesium to a lesser extent.

The changes in total and soluble calcium

Table 3. Pectin analysis on fresh pears.

Harvest date	Firmness	Pectin total % [a]	Protopectin % [a]	Protopectin % of Total	Vers.-sol. % [a]	Vers.-sol. % of Total	Vers.-sol. % of Proto	Vers.-insol. % [b]	Vers.-insol. % of Proto	Water-soluble % [b]	Water-soluble % of total	Marc extracted with acid alcohol (%)
6-30	23.3	.966	.430	44.4	.172	17.7	40.0	.258	60.0	.536	55.5	6.6
7-7	21.8	.925	.443	47.8	.234	26.3	52.8	.209	47.1	.482	52.0	6.5
7-14	21.2	.895	.485	53.0	.237	14.2	26.2	.358	73.8	.410	45.7	5.3
7-21	20.5	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
7-28	23.5	.870	.425	48.7	.092	10.5	21.6	.333	78.3	.445	51.0	5.1
8-4	19.1	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
8-12	17.6	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
8-18	16.0	.745	.430	57.6	.114	15.3	26.5	.316	73.5	.315	42.5	4.3
8-25	14.6	.820	.528	64.2	.161	19.6	30.5	.367	69.5	.292	35.5	4.5
9-1	13.2	.650	.475	73.0	.140	21.6	29.6	.334	70.5	.175	26.9	3.9
Coefficient of correlation [c]		0.945	0.580	0.941	.....	.....	.....	.....	.....	.....	0.948	0.829

Harvest date	Firmness	% Pectin as AUA on fresh basis	Analysis in pure pectin basis: % methoxyl	% acetyl	Degree of esterification (%)
6-30	23.3	.966	7.7	9.8	65.2
7-7	21.8	.925	9.1	7.0	70.0
7-14	21.2	.895	8.7	5.8	68.8
7-21	20.5	.....	.....	.....	.....
7-28	23.5	.870	7.4	9.9	66.0
8-4	19.1	.....	.....	.....	.....
8-12	17.6	.....	.....	.....	.....
8-18	16.0	.745	7.7	7.8	61.5
8-25	14.6	.820	8.4	5.8	62.5
9-1	13.2	.650	9.4	6.9	64.2
Coefficient of correlation [c]		0.945	−0.427	0.574	0.660

[a] Independent determination.
[b] By difference.
[c] Firmness the independent variable.

Table 4. Changes in calcium and magnesium content in pear slurry and slurry filtrate after cold storage and subsequent ripening.

Sample [a]	Storage conditions (°F)	Firmness	Calcium (ppm)		Magnesium (ppm)		Ratios Mg/Ca	
			Slurry	Filtrate	Slurry	Filtrate	Slurry	Filtrate
A-1	Fresh	15.2	39	25	66	51	1.7	2.0
A-2	9 da 41°, 3 da 68°	6.0	32	22	61	63	1.9	2.9
A-3	9 da 41°, 6 da 68°	1.4	33	27	76	69	2.3	2.6
		Av.	35	25	68	61	2.0	2.5
B-1	Fresh	20.0	40	13	102	55	2.5	4.2
B-2	11 da 41°,	18.9	....	17	....	63	....	3.7
B-3	11 da 41°, 4 da 68°	3.3	31	....	94	....	3.0	....
B-4	11 da 41°, 7 da 68°	1.6	35	28	86	78	2.5	2.8
		Av.	35	19	94	66	2.7	3.6
C-1	Fresh	17.7	77	28	75	55	.98	2.0
C-2	7 da 68°	1.6	87	46	90	81	1.03	1.8
		Av.	82	37	82	68	1.01	1.9
D-1	30 da 41°	13.6	64	26	88	68	1.4	2.6
D-2	30 da 41°, 3 da 68°	1.9	49	30	83	78	1.7	2.6
		Av.	56	28	85	73	1.5	2.6
E-1	60 da 32°	16.9	82	35	94	69	1.1	2.0
E-2	60 da 32°, 4 da 68°	1.5	85	46	85	79	1.0	1.7
		Av.	83	40	89	74	1.0	1.8

[a] A and B series, fruit grown near Davis; C, D, and E series, fruit grown near Placerville.

content in maturing fruit are shown in Fig. 2, and those for magnesium are shown in Fig. 3. The changes in percent insoluble calcium and magnesium content are shown in Fig. 4, and the changes in the ratio of Mg/Ca are shown in Fig. 5. In Tables 2, 3, 4, and 5, the independent variable is time of harvest.

Table 3 shows the pectin analyses of the marcs prepared from fruit harvested at various stages of maturity. McCready and McComb (1954) reported similar changes in the marc of two samples of pears (unripe and ripe Bartlett) prepared by slicing the fruit into boiling alcohol. They reported, however, that a considerable amount of pectin present from ripe pears dissolved in this alcoholic solution. The yield of pectin extracted from the marc prepared from unripe pears with 0.5% Versene at pH 6 at 25°C, was 37% of the total present, compared with 60% from marc of ripe pear. The degree of esterification of the extracted pectin was 89% in unripe and 45% in ripe pears, and the acetyl content was 3.3% in ripe and 3.7% in unripe pear pectin. Subsequently, however, Gee *et al.* (1959) reported that the percent esterification of pectin in Bartlett pears increased from 85% in green hard pears to 95% in green mature pears, and decreased during ripening to 64%.

The data in Table 3 show, for fruit maturing on the tree, that the total pectin and the water-soluble pectins decrease and the protopectin increases in both actual value and as percent of total as shown by the negative coefficient of correlation. In his Table 2 Weurman reported a similar rise in protopectin in maturing fruit. The acetyl content and the degree of esterification decrease with maturation, in agreement with published data, although the correlation is below the minimum significance. Methoxyl content increases on maturation.

In the detached ripening fruit, significant metabolic changes occur (Tables 4–6). While the total pectin also decreases the protopectin is now broken down and appears in a water-soluble form. The degree of es-

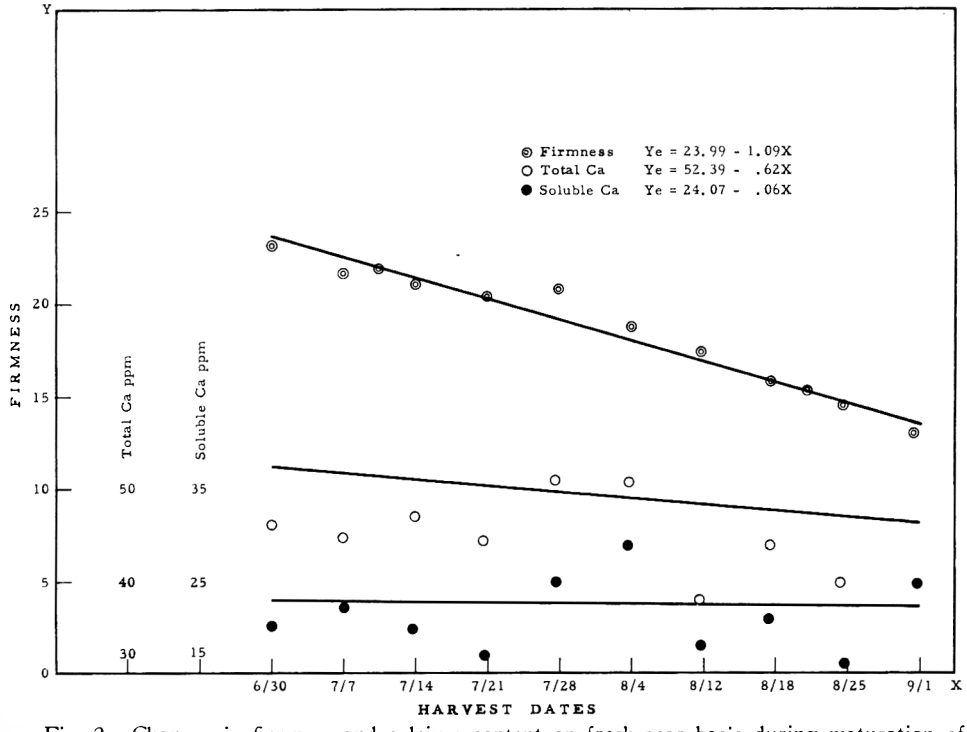

Fig. 2. Changes in firmness and calcium content on fresh-pear basis during maturation of pears on tree.

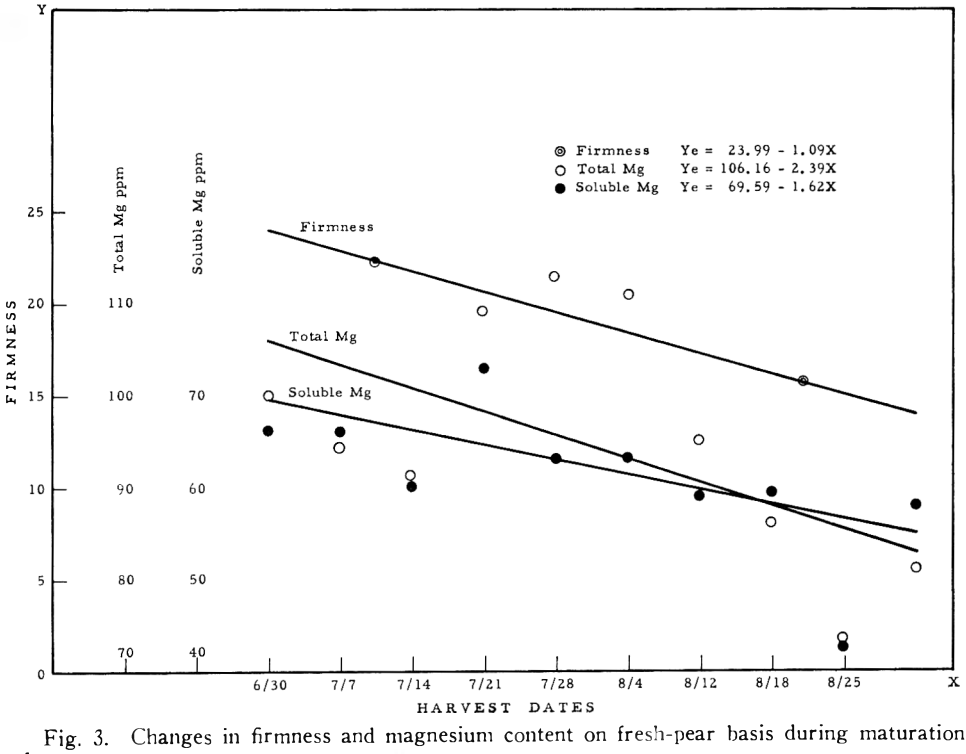

Fig. 3. Changes in firmness and magnesium content on fresh-pear basis during maturation of pears on tree.

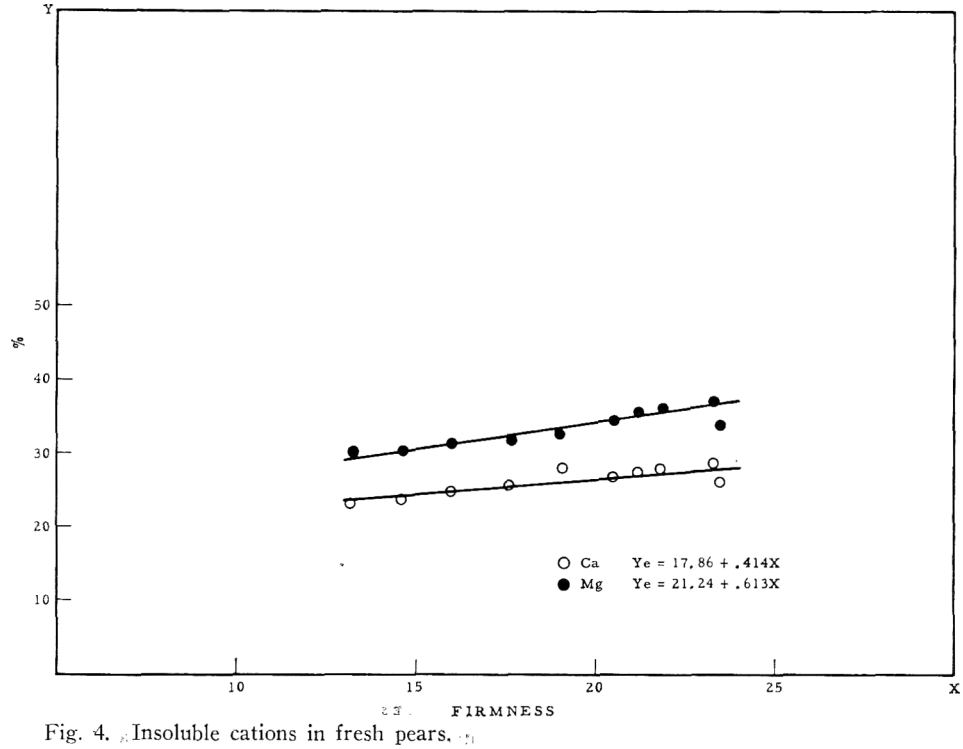

Fig. 4. Insoluble cations in fresh pears.

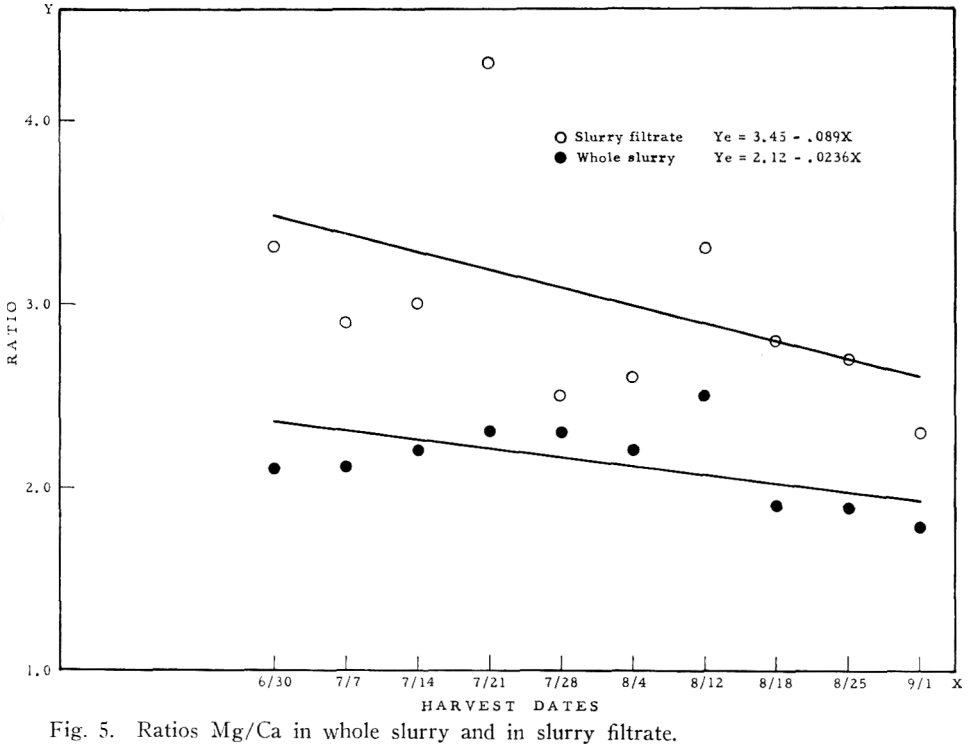

Fig. 5. Ratios Mg/Ca in whole slurry and in slurry filtrate.

Table 5. Relationships between percentages of water-insoluble calcium and magnesium and protopectin content.

Sample	Firmness	Insoluble Ca as % of total	Insoluble Mg as % of total	Protopectin as % of total Pectin
A-1	15.2	35.9	22.7	40.5
A-2	6.0	31.2	0.0	27.4
A-3	1.4	18.2	9.2	15.5
B-1	20.0	67.4	46.0	26.5
B-4	1.6	20.0	9.3	7.0
C-1	17.7	62.6	26.7	40.1
C-2	1.6	44.9	10.0	7.3
D-1	13.6	59.4	21.7	44.9
D-2	1.9	38.7	6.2	9.2
E-1	16.9	57.4	26.6	49.3
E-2	1.5	45.9	7.1	18.0

	Coefficients of correlation		
	Insoluble Ca	Insoluble Mg	Protopectin
Combined series A & B	0.921	0.877	0.725
Combined series C, D, & E	0.942	0.990	0.947

Table 6. Pectin changes on ripening in storage expressed as percent of anhydrouronic acid on fresh-fruit basis.

		Coefficients of correlation					
		Pectin					
Treatment (°F)	Firmness	Total	Proto-	Water-sol.	Methoxyl	Acetyl	% esterification
Initial 1957	15.2						
9 da 41°	13.7						
9 da 41°, 2 da 68°	6.0	.869	.991	−.205	.743	−.817	.832
9 da 41°, 5 da 68°	1.4						
Initial 1958	20.0						
11 da 41°	18.9						
11 da 41°, 4 da 68°	3.3	.894	.961	−.160	.744	−.909	.825
11 da 41°, 7 da 68°	1.6						

Table 7. Titrimetric analysis of marc.

Firmness	23.3	21.8	21.2	23.5	16.0	14.6	13.2
Free acidity							
ml 0.1N NaOH	2.35	2.60	2.60	2.85	3.30	3.30	3.50
mg AUA	41.3	45.7	45.7	50.2	58.2	58.2	61.5
Total esters							
ml 0.1N NaOH	10.90	11.80	12.20	13.25	13.60	14.30	13.85
mg AUA	192	208	215	233	239	252	244
% esterification	82.3	82.0	82.5	82.5	80.5	81.2	80.0
% AUA in marc	23.33	25.37	26.07	28.32	29.72	31.02	30.55
% pectin (fresh fruit)	1.53	1.65	1.38	1.22	1.28	1.34	1.19

terification also decreases, as does the methoxyl content, but the acetyl content rises.

No significant changes in cations are apparent in fruit while on the tree and when ripened in storage. The behavior of calcium is similar in both instances, but magnesium becomes rapidly soluble as shown by a high degree of correlation in the latter instance and a poor one for fruit on the tree (Table 13, I and II).

To obtain additional information on the free and esterified carboxyl groups of the pectins in the marc, the free acidity and total acidity of the marcs were determined by direct titration of the acid-washed acid-free marc.

The results in Table 7 are expressed in ml of 0.1*N* NaOH per g of marc, and are calculated as mg anhydrogalacturonic acid. These values indicate a fairly constant degree of esterification but an increase in pectin content of the marc during maturation of the fruit. The calculated pectic content on a fresh-fruit basis decreases as firmness decreases. The values determined by titration are considerably higher than those determined by the colorimetric Versene-pectinase extraction procedure. The correlation with the carbazole method is poor, 0.748.

On the basis of these high values, the weight of pectin present in green fruit is 1.53 g per 100 g. If this is 80% esterified, there would be available for cation binding 1.74 meq of carboxyl groups from the anhydrogalacturonic acid residue present. The total calcium and magnesium present in the sample respectively amount to 2.30 and 4.12 meq. Of this, 1.13 and 1.52 meq are insoluble. The total 2.65 meq of insoluble calcium and magnesium cannot be "bound" by the available 1.74 meq of pectin carboxyl groups, and much less so when the lower carbazole values are considered. Even with the most mature fruit, the same condition prevails.

It is unlikely that protopectin exists as chains of polygalacturonic acid ionically bound to each other by Ca^{++} ion bridges (Henglein, 1943), as quite clearly shown by Deuel *et al.* (1950). The data subsequently published by Haas-Schulz (1951) do not refute the conclusions of Deuel *et al.* (1950) but merely add information on the composition of calcium pectinate. Even less likely would be double salt formation. Double salts of galacturonic acid do occur, but they are not formed by either di-galacturonic acid or tri-galacturonic acids (Phaff and Luh, 1952).

Joslyn and Deuel (1962) recently observed that the extractability of pectins from green firm apples varies markedly with the method used to prepare marc. From marc prepared by blending apple flesh with alcohol and inactivating enzymes present by heating the alcoholic mixture at 80°C under reflux, practically no water-soluble pectins were obtained by extraction with distilled water at 20°C. From marcs prepared by the procedure of Gee *et al.* (1958), the quantities obtained were considerable. After extraction with water, further pectins were not extractable with Versene at pH 6, as used by McCready and McComb (1952). Blending apple flesh, previously dehydrated with alcohol, with acidified alcohol was not equivalent to washing alcohol-insoluble solids with acidified alcohol. Apple marcs practically ash-free were not found to contain appreciable water-soluble pectins, as would be expected if the insoluble pectins were present as calcium salts.

Marc prepared by treatment with acidified alcohol, as outlined under "Preparation of marc," is practically free of calcium and magnesium salts in comparison with fresh pear tissue. The fresh pear tissues have the composition shown in Table 8.

The marc after extraction with alcoholic, approximately 0.6*N*, hydrochloric acid had the analysis, representing the composite of seasonal samples, shown in Table 9.

The relatively high phosphorus content of

Table 8. Composite analysis of fresh pears (10 harvests).

	On fresh basis (ppm)
Calcium	53
Magnesium	94
Phosphorus	119
Potassium	1246
Sodium	4
Ash	3900
Marc content	5.08%
Acid-washed marc content	4.08%

Table 9. Mineral analysis of composite acid-washed seasonal samples.

	In marc (ppm)	Fresh basis (ppm)	% Loss
Calcium	310	15.5	70.7
Magnesium	50	2.5	97.4
Phosphorus	376	18.9	84.0
Potassium	218	10.9	98.2
Sodium	38	1.9	52.5
Iron	80	4.0	–
Ash	3300	115	97.0

pear marc is not surprising. Henglein *et al.* (1949) reported that the phosphate content of pectins prepared from apple pomace can range from 0.51 to 0.04% as P_2O_5, depending on the method of purification. However, they considered that only about 0.004 to 0.008% of phosphate could be firmly bound by pectin.

To obtain additional information on the distribution of phosphorus in pear tissue, the pear marc stored in alcohol was fractionated into storage alcohol, alcohol-insoluble solids, acid-washed alcohol-insoluble solids, and acidic wash solution. The storage alcohol and acidified alcohol extracts were evaporated and ashed, as well as the marc and the acid-washed marc. The ashed materials were analyzed for cations, phosphorus, and nitrogen, with the results shown in Table 10. The data indicate that most of the calcium present in pear tissue was insoluble in alcohol but soluble in acidified alcohol (i.e., present as ionically bound). In contrast, most of the magnesium and potassium was soluble. Of the total phosphorus content, most was found in the marc but much of this was acid-soluble, indicating that it was either loosely held or combined as calcium or magnesium phosphates.

The high iron content in the marc extracted with acidified alcohol is unexpected since it does not originate from equipment and the amount in the marc can be reduced by increasing the time of extraction.

Although cation-sequestering agents like Versene are used widely in pectin extraction (Owens *et al.*, 1952), their value may not be in sequestering calcium from calcium pectinates, since the sequestering property depends on the pH of the medium. Ammonium oxalate is weak, but at higher temperatures (80°C or above) heat and acidity may be the major solubilizing factors. McCready and McComb (1952) reported that extraction of pectins with Versene at pH 7 were complete only on prolonged heating.

The relative insolubility of the cations and phosphorus in the marc of pears was determined by extraction of a 0.5-g portion with 100 ml of deionized water for 4 hr while stirring on a magnetic stirrer. The mixture was then centrifuged and the residue again extracted to make 8, 24, and 48 hr of extraction time. Another sample was extracted as above but with one teaspoon of washed Amberlite 120 (H). Each of the extracts was evaporated, ashed, and analyzed. The resin was separated from the marc before ashing. The pH of the water extracts increased from 3.6 after the first extraction period to 4.1, 4.7, and 5.4, respectively, for the succeeding extractions. The pH of the extracts in the presence of resin varied from 3.6 to 4.0, 4.4, and 5.0, respectively. The extracts and residues had the analyses shown in Table 11.

These values indicate that water extracts 18% of the calcium, 20% of the magnesium,

Table 10. Calcium, phosphorus, and nitrogen content of marc and marc extracts in mg per 9.59 g of marc or 7.21 g of acid-washed marc (equivalent to 250 g of original pear tissue).

	Marc	Storage alcohol	Acid-washed marc	Acid-wash solution
Calcium	26.6	3.40	4.35	5.60
Magnesium	10.1	11.00	0.35	2.75
Potassium	306.0	307.0	4.05	50.50
Sodium	2.0	3.45	0.85	2.00
Nitrogen (%)	0.66	.....	0.62	.....
Phosphorus				
Total	52.6	23.25	18.00	4.45
Inorganic	0.02	1.93	0.00	0.15

Table 11. Mineral analysis of ion-exchange-treated marc (ppm).

Sample	Ca	Mg	P	K	Na
Water extract	40	20	25	130	60
Residue	180	80	180	210	80
Resin extract	40	20	20	150	60
Residue	130	80	158	210	80

12% of the phosphorus, 33% of the potassium, and 43% of the sodium. The resin had no effect on extraction of these constituents. The lower values for resin residue are accounted for by a small loss of solids during separation of the solids from resin.

Tables 4 and 5 show the changes in calcium and magnesium in pears held in cold storage at 41°F, and then ripened at 68°F. Graphically these changes are shown in Figs. 6 and 7. Comparable results for freshly harvested fruit are taken from Table 2 and are shown in Fig. 4. When firmness is the independent variable the correlation with the insoluble cations is good for fresh fruit as well as for fruit ripened off the tree (Table 13, I and II). Comparison of Fig. 4 with Figs. 6 and 7 suggests that calcium is affected somewhat more by the change in metabolic activity in the detached fruit than magnesium, and it is solubilized more rapidly with decreasing firmness. Numerically, this behavior of insoluble calcium is expressed in a somewhat higher correlation, as shown in Table 13, I and II. The coefficient of correlation for insoluble Ca is 0.908 for fresh fruit and 0.921 and 0.942 for both series of detached fruit. The correlation between protopectin, cations, and firmness is also higher for detached fruit. These analytical differences between fruit growing and in storage have been observed with other fruit. Thus, though the correlation between firmness and cellulose is high in different varieties of apples, such correlation was not found during softening and storage (Kertész *et al.*, 1958). The data available on stored pears are too meager for actual comparison, though important differences in behavior are indicated.

The coefficients of correlation for total pectin, protopectin, water-soluble pectin, methoxyl, acetyl, and degree of esterification were calculated with firmness used as the independent variable. These data were calculated for freshly harvested pears as well as

Table 12. Pectin changes on ripening in storage expressed as percent of anhydrouronic acid on fresh-fruit basis.

Time and conditions of storage (°F)	Firmness	Coefficients of correlation					
		Pectin					
		Total	Proto	Water-sol.	Methoxyl	Acetyl	% esterification
Storage at 41°							
Initial	17.7						
7da 68°	1.6						
20 da 41°, 0 da 68°	13.6						
20 da 41°, 3 da 68°	1.9						
30 da 41°, 0 da 68°	6.6	.867	.983	−.940	.736	−.692	.703
30 da 41°, 3 da 68°	1.6						
40 da 41°, 0 da 68°	2.6						
40 da 41°, 2 da 68°	1.7						
50 da 41°	1.3						
Storage at 32°							
Initial	17.7						
7 da 68°	1.6						
30 da 32°, 0 da 68°	17.9						
30 da 32°, 5 da 68°	1.6	.779	.881	−.739	.710	−.451	.732
60 da 32°, 0 da 68°	16.9						
60 da 32°, 4 da 68°	1.5						
90 da 32°, 0 da 68°	11.6						
90 da 32°, 8 da 68°	1.5						

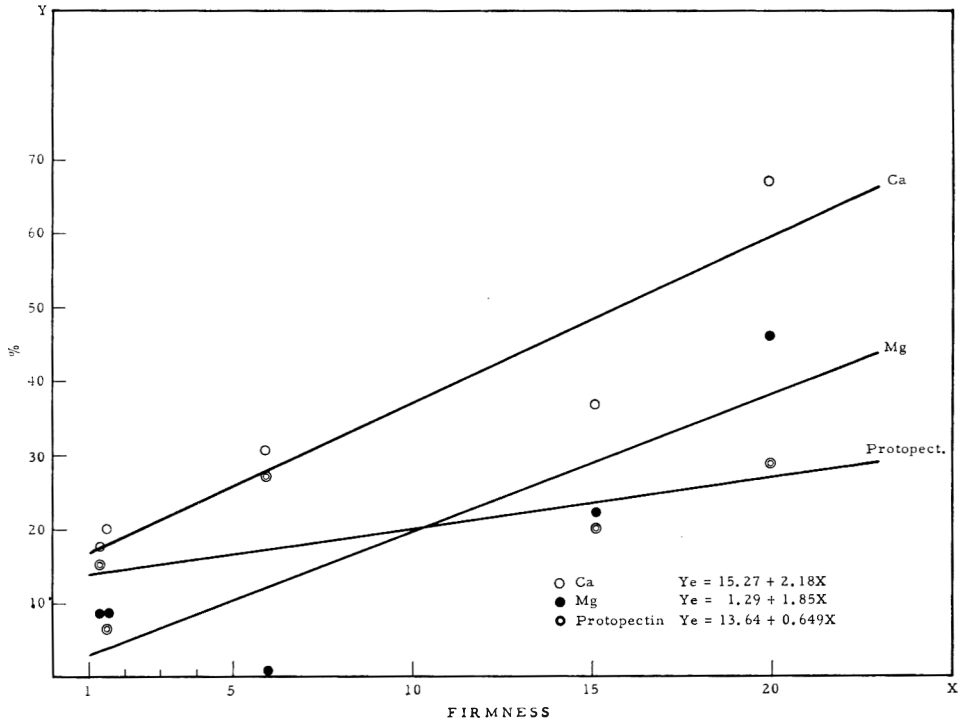

Fig. 6. Relation between firmness, insoluble cations, and protopectin in ripening detached pears. A–B series.

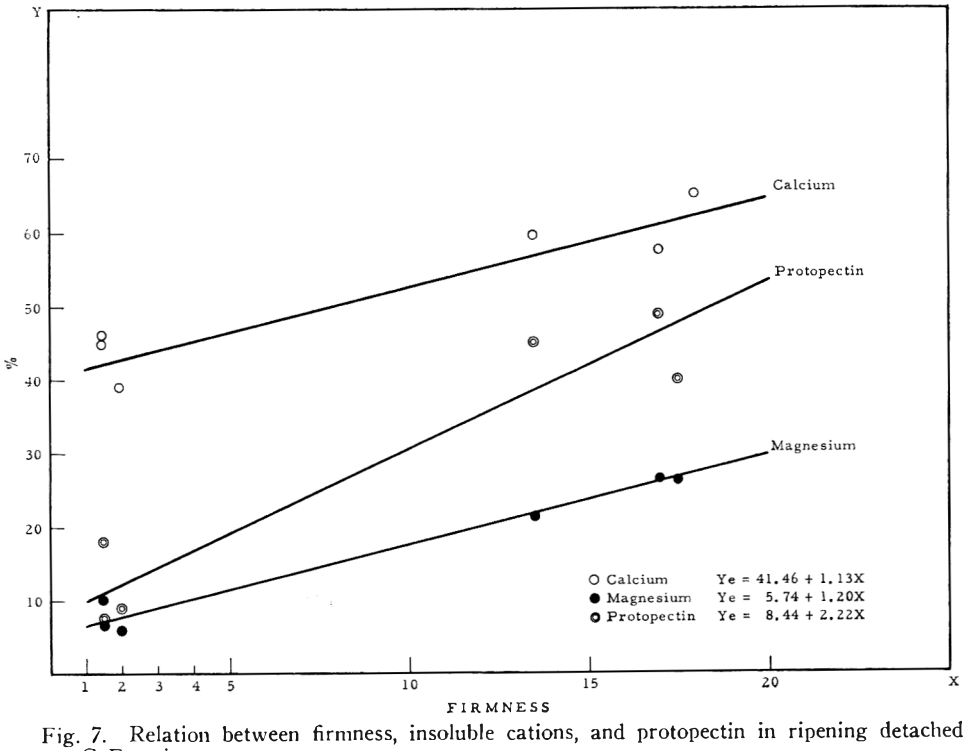

Fig. 7. Relation between firmness, insoluble cations, and protopectin in ripening detached pears. C–E series.

Table 13. Summary of coefficients of correlation at 5% confidence level, firmness being the independent variable.

Relationship of calcium and magnesium to firmness of pears

I. Fruit maturing on tree (from Table 2)

Calcium		Magnesium	
Total	0.913	Total	0.909
Insoluble	0.908	Insoluble	0.920
Soluble	0.852	Soluble	0.913
Minimum significance	0.610	Minimum significance	0.610

II. Fruit in storage (from Tables 4 and 5); fruit held in cold storage and subsequently ripened

	Series A-B	Series C, D, and E
Calcium		
Insoluble	0.921	0.942
Soluble	−0.265	−0.468
Magnesium		
Insoluble	0.877	0.990
Soluble	−0.045	−0.663
Protopectin	0.725	0.947
Marc	0.991	0.783
Minimum significance	0.811	0.811

Relationship of pectin fractions and pectin composition to firmness of pears

III. Fruit maturing on tree (from Table 3)

Total pectin	0.945	
Protopectin	−0.941	as % of total pectin
Protopectin	−0.580	as % on fresh fruit basis
Water-soluble	0.948	as % of total pectin
Methoxyl	−0.427	
Acetyl	0.574	
Esterification %	0.660	
Marc	0.829	
Minimum significance	0.610	

IV. Fruit in storage (from Table 6); fruit held in storage and subsequently ripened

	1957	1958	
Total pectin	0.869	0.894	
Protopectin	0.991	0.961	as % of total pectin
Water-soluble	−0.205	−0.160	as % of total pectin
Methoxyl	0.743	0.744	
Acetyl	−0.817	−0.909	
Esterification %	0.832	0.825	
Marc	0.291	0.170	
Minimum significance	0.811		

V. Fruit in storage (from Table 12); fruit held in cold storage and subsequently ripened

	Storage at 41°F	Storage at 32°F
Total pectin	0.867	0.779
Protopectin as % of total pectin	0.983	0.881
Water-soluble as % of total pectin	−0.940	−0.739
Methoxyl	0.736	0.710
Acetyl	−0.692	0.451
Esterification %	0.703	0.732
Marc	0.828	0.812
Minimum significance	0.666	

pears stored at 32°F as long as 90 days and at 41°F for as long as 50 days and subsequently ripened. The coefficients of correlation for fresh pears are shown in Table 3, and those for pears ripened after cold storage are in Tables 6 and 12.

The metabolic processes apparently follow different paths in pears maturing on the tree and in detached fruit. In fruit on trees, protopectin and methoxyl show negative correlation, i.e., values increase with decreasing firmness; in detached fruit the correlation is positive. Water-soluble pectin shows a reverse behavior: It increases in ripening detached fruit and decreases in fruit maturing on the tree. Analytical data that were used for Tables 6 and 12 will be published at a later date.

The similarities and the differences in metabolic transformations occuring in pears maturing on the tree and in mature pears ripening in storage are summarized in Table 13.

In group I is shown the relationship of calcium, magnesium, and firmness. The high degree of correlation suggests that both cations are equally important participants in the firmness of fruit. The total amount of cations was determined on a separate aliquot from that used for determination of soluble and insoluble fractions.

In group II are shown the metabolic changes in pears held at low temperature and then ripened. Both series A-B and C-E are essentially similar between themselves, but different from similar metabolic changes occurring on the tree. A negative correlation indicates an increase of soluble forms during ripening.

Groups III, IV, and V show the profound changes that occur in the metabolism of pectin when the fruit is removed from the tree. Total pectin decreases whether the fruit is on the tree or in storage. The great change in the detached fruit occurs in protopectin and in water-soluble pectin. The protopectin decreases are shown in groups II, IV, and V. The correlation is highly significantly negative in group III, and highly significantly positive in groups II, IV, and V. For water-soluble fraction, the correlation is reversed in detached fruit, indicating a rapid solubilization of pectin when the fruit ripens. Methoxyl and acetyl show a change similar to that of protopectin. The degree of esterification decreases when fruit matures, and the correlation is better in detached fruit. The marc content shows a high degree of correlation in all instances except in group IV, where the time was too short for a significant change to occur.

Data recently accumulated indicate that changes in pectic constituents are not correlated closely with firmness, at least for apple and tomato fruit. Thus, Reeve *et al.* (1953a,b) found little or no relation between pectin content and the texture of apples. This was confirmed by Kertész *et al.* (1958) and Woodmansee *et al.* (1959). Kertész *et al.* (1958) reported good correlation between firmness and cellulose or alcohol-insoluble solids content. Woodmansee *et al.* (1959) reported a close negative correlation between firmness and alcohol-insoluble solids during the maturation of apple fruit, but found no consistent relation between ripening and soluble pectin content. Doesburg (1957) reported little change in molecular weight and degree of esterification of pectin during the ripening of apples, but found an increase in soluble pectin content with a decrease in soluble calcium content. Jermyn and Isherwood (1956) reported no change in the galacturonic acid content of alcohol-insoluble solids of pears during ripening, a marked increase in araban, smaller increases in glucosan and galactan, and a decrease in xylan.

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Tenderness of Beef.
IV. Relations of Shear Force and Fiber Extensibility to Juiciness and Six Components of Tenderness [a]

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SUMMARY

Shear-force values were markedly and significantly higher at meat temperatures of 80°C (well-done) than at 61°C (rare) in longissimus dorsi (LD) steaks, but only slightly higher in biceps femoris (BF) steaks from 180 animals. Neither muscle showed significant differences between shear-force values at 80 and 100°C. The two muscles had similar shear-force values at 61°C, although the connective tissue scored much tougher in BF than in LD steaks. Correlations were low between shear-force values and panel scores for tenderness of connective tissue. The highest coefficients between shear-force values and panel scores were in LD cooked to 80 and 100°C—for softness to tooth pressure, ease of fragmentation, and adhesion. These indicate that low scores (tough meat) were associated with high shear-force values. Correlations for all possible combinations of shear-force values for the two muscles and the three conditions of cooking were made. Within LD all coefficients were significant and high, which indicated that ranking of the animals for shear-force value was similar at all temperatures within this muscle. Within BF the only high coefficient was for 80 vs. 100°C. Those involving the two muscles together were not high and not always significant. Thus, shear-force values of one muscle would not be satisfactory for indicating the tenderness of another muscle in the same carcass. Variation was found among the various core positions within a steak.

Extensibility of muscle fibers was greater at 100 than at 61°C. This was significant in LD but frequently not significant among the lots in BF. Extensibility was rather closely related to shear-force values at both temperatures in LD, but only at 100°C in BF. Availability of muscle fibers long enough for these determinations (5 mm) varied widely in BF. This availability in the BF steaks cooked to 100°C appeared to be related to scores for mealiness.

INTRODUCTION

Objective tests for tenderness have received much attention because such tests have been considered to be more reliable (repeatable) than panel scores. Various mechanical devices have been developed to measure tenderness and its components (Black *et al.*, 1931; Tressler *et al.*, 1932; Tressler and Murray, 1932; Volodkevich, 1938; Winkler, 1939; Steiner, 1939; Kramer *et al.*, 1951; Hurwicz and Tischer, 1954; Miyada and Tappel, 1956; Proctor *et al.*, 1956; Sperring *et al.*, 1959; Pilkington *et al.*, 1960). The one used most widely by research workers is the Warner-Bratzler shearing device described by Black *et al.* (1931), or some modification of it. Some workers have reported good agreement between it and panel scores for tenderness, whereas others have found poorer agreement. Some of these coefficients of correlation for beef were −0.9

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for 50 muscles cooked in lard at 250°F (Ramsbottom and Strandine, 1948); −.369 for steaks oven-cooked at 450°F and −.404 for braised steaks (Paul *et al.*, 1956); and respectively −.554, −.001, and −.715 for longissimus dorsi (LD) steaks oven-cooked to 55, 70, and 85°C (Visser *et al.*, 1960).

The success of a mechanical device for measuring various components of tenderness depends on the closeness of its relation to human impressions of tenderness. One score for tenderness is a composite of many mouth impressions, and agreement between the results from a mechanical device and this score may be close when certain components are involved but less close or even poor when other components are involved. The data for six components of tenderness (Cover *et al.*, 1962a,b,c) as well as for shear-force values on the same steaks afforded an opportunity to investigate the relation of shear-force values to these components—softness to tongue and cheek, softness to tooth pressure, ease of fragmentation and mealiness of muscle fibers, adhesion between muscle fibers, and tenderness of connective tissue.

Extensibility of muscle fibers has been shown to be related to tenderness by Wang *et al.* (1956) and by Hostetler and Cover (1961). Further work with six components of tenderness on a rather large number of animals should provide a basis for better understanding of this relation.

EXPERIMENTAL

Cover *et al.* (1962a) described the cooking and sampling methods, the animals, plan for utilizing the meat from the two muscles, scoring sheet used by the panel, outlines of the analyses of variance, and pooling of the lot variances and covariances to obtain a single coefficient for each pair of variables. Additional analyses of variance for shear-force values within each muscle were made for each of the nine lots (Table 1). This made it possible to test for homogeneity of tenderness (shear-force values) within the muscle and for possible interaction between core portions and final cooking temperatures. Panel scores for these steaks were reported for tenderness of connective tissue (Cover *et al.*, 1962a), for juiciness and the two softness components of tenderness (Cover *et al.*, 1962b), and for the muscle fiber components (Cover *et al.*, 1962c).

Shear-force values were obtained on 1-in. steaks from LD and BF muscles cooked to 61 and 80°C by dry heat and to 100°C and held there for 25 min. in moist heat. They were obtained for all of the 180 animals. A ½-in. core from each quarter of the steak was sheared with the Warner-Bratzler device. Since a steak is not homogeneous for tenderness, the average from these four cores was used as the value for the steak. A great deal of care was taken to obtain the cores along and not across the fibers. This was done so that the orientation of muscle fibers during shearing would be similar for all cores.

Extensibility measurements were made on muscle fibers obtained from a small area from one section of the sheared cores, but only from those steaks cooked to 61 and 100°C. The procedure was that described by Hostetler and Cover (1961). These measurements were made on LD and BF steaks from only 128 of the 180 animals because of lack of time. Selections were made on the basis of those sires and sexes for which this information would have most value.

RESULTS AND DISCUSSION

Shear-force values. Comparisons of temperatures within muscles may be made within each lot (Fig. 1). In LD the shear-force values were significantly higher in

Table 1. Outline of analyses of variance.

Variables	Degrees of freedom	Error terms
Total	(a)	
Treatments	11	Animals
Core position	(3)	(b)
Temperatures	(2)	(b)
Core position × temperature	(6)	Animals
Animals within core position and temperature	(a)	

(a) These degrees of freedom vary with the lot.
(b) These error terms were core × temperature if core × temperature was significant. If core × temperature was not significant, "animals" was used as the error term.

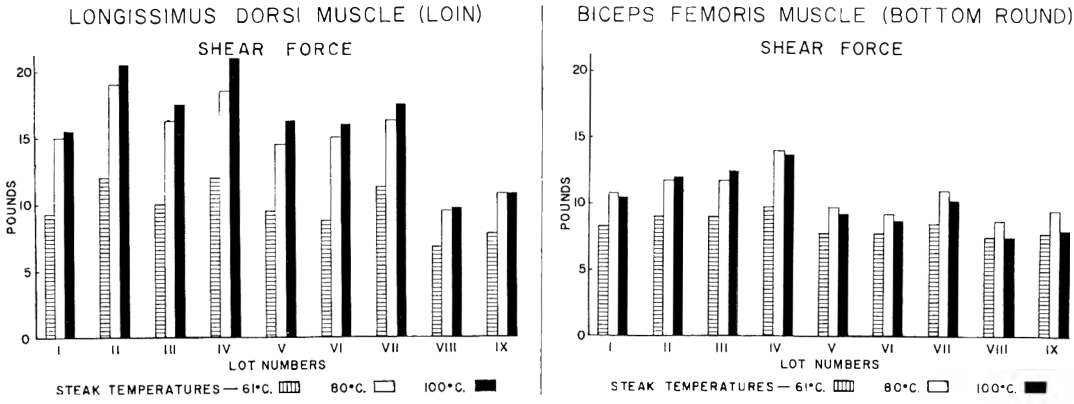

Fig. 1. Average shear-force values for steaks cooked to three internal temperatures.

each lot at 80°C (well-done) than at 61°C (rare). But at 100°C the increase above that at 80°C was small and not significant in any lot. These trends indicate greater toughening at 80 than 61°C, with little if any further toughening at 100°C. A similar pattern of response to temperatures in panel scores for LD was found only in softness to tooth pressure (Cover *et al.*, 1962a,b,c). These trends indicate that shear-force values in LD may be detecting the tightening of the network of the protein structure during denaturation of the meat and may be related to dehydration of the muscle fiber. In BF also, shear-force values at 80 were higher than at 61°C, but so slightly as to be significant only in lots 1, 4, and 9. The shear-force values at 80 and 100°C were not significantly different in any lot. The muscle fiber components also did not tender or toughen alike in the two muscles in response to these three temperatures (Cover *et al.*, 1962b,c).

Trends with meat temperatures of 61 vs. 80°C in both muscles were toward toughening at the higher temperature with shear-force values, but were toward tendering with scores for connective tissue and with collagen content (Cover *et al.*, 1962a). With meat temperatures of 80 vs. 100°C in LD, neither shear-force values nor scores for the already tender connective tissue changed greatly, although collagen content decreased. In BF at 80 and 100°C, the shear-force values also did not change greatly although the connective tissue scores and collagen content indicated marked tendering. Therefore, shear-force values obtained across the grain of the meat would appear to be unreliable as a measure of the tendering of connective tissue by heat or as a means of relating heat changes in collagen to tenderness of connective tissue. Such complicated patterns for connective tissue and muscle fiber components of tenderness cannot be detected in shear-force values alone, and proof that they are involved in shear-force values is difficult.

When the two muscles were compared, there was no significant difference in shear-force values at 61°C, which seems strange because of the much tougher connective tissue and the much higher collagen content in BF (Cover *et al.*, 1962a). At 80°C, LD with the more tender connective tissue and the less collagen content had significantly higher shear-force values (tougher meat). At 100°C, where the two muscles did not differ greatly in scores for connective tissue or in collagen content, the difference in shear-force values was most pronounced. Similar results have been reported by Cover and Hostetler (1960). Thus, shear-force values obtained across the grain of the meat would be unreliable as a measure of the variation in tenderness of connective tissue among muscles or as a means of relating collagen content in different muscles to the tenderness of their connective tissues. Patterns between muscles somewhat similar to those in shear-force values occurred only in scores for ease of fragmentation and lack of adhesion. These are characteristics of the muscle fibers.

Table 2 shows interrelations between shear-force values and each of the panel scores within a muscle and condition of cooking. Within LD, the coefficients indicate that at 80 and 100°C the shear-force values were closely associated with ease of fragmentation and with adhesion as well as with softness to tooth pressure. The negative coefficients indicate that the tougher meat was associated with the higher shear-force values. In BF, none of the coefficients was really high, but at 100°C, where the effect of connective tissue is at a minimum, the highest were for ease of fragmentation, adhesion, softness to tooth pressure, and mealiness. Shear-force values were not closely correlated with tenderness of connective tissue in either muscle.

Meat to be called "tender" by the consumer must require little chewing either to break it apart or to get it ready for swallowing. If this definition of tenderness is accepted, there are a number of conditions under which meat may be regarded as tender. Shear-force as a measure of tenderness seems to be related to certain of these conditions but not to others. 1). Cooked meat with a large amount of unchanged collagen in its connective tissue would be called tough, yet shear-force does not appear to measure it accurately. 2). Meat cooked rare is soft and juicy and may be swallowed with little chewing, even when the muscle fibers adhere tightly and do not fragment easily. This kind of "tenderness" is not related to shear-force. If, however, this same piece of meat is chewed well, as our judges are instructed to do, the relative tenderness of the muscle fibers will be related to shear-force values of the meat. In well-done meat also, the ease of fragmentation of muscle fibers and adhesion between muscle fibers is related to shears. 3). Meat cooked well-done is less juicy than rare and is firm, or even hard, to tongue and cheek and may be hard to tooth pressure also.. Shear-force seems to follow rather well the change in hardness (toughening) in meat brought about by cooking from rare to well-done. Whether hardness to tooth pressure, ease

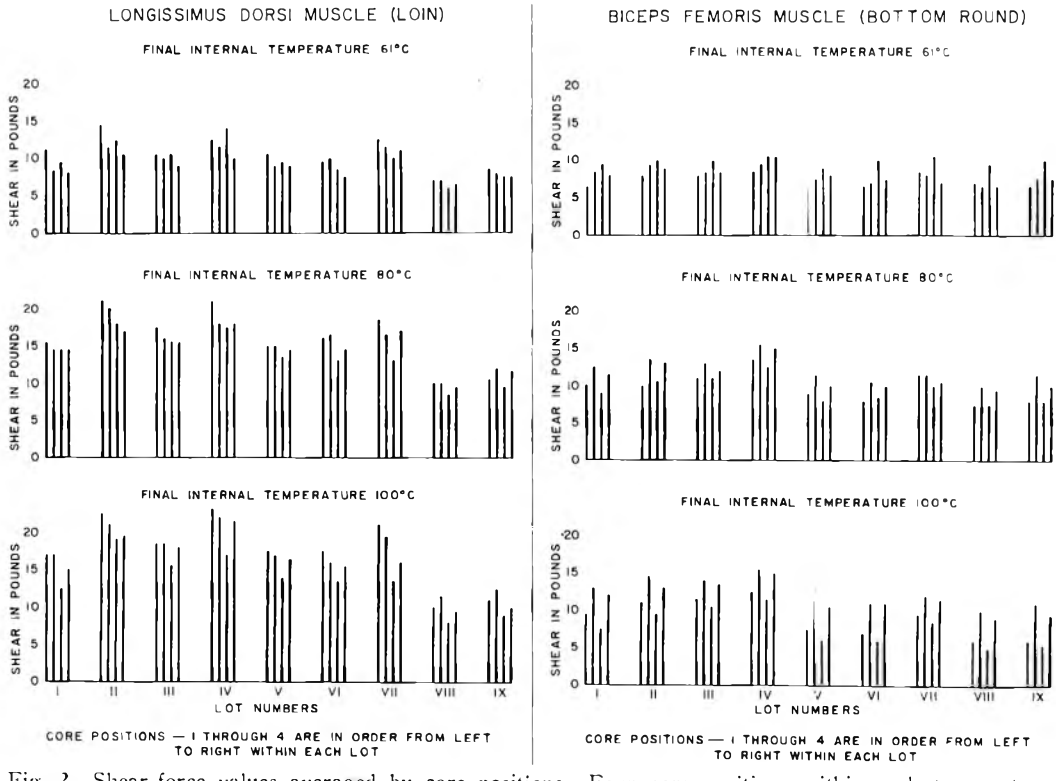

Fig. 2. Shear-force values averaged by core positions. Four core positions within each temperature.

Table 2. Pooled correlations calculated within lots.

Variables [a]	Pooled coefficients					
	Longissimus dorsi			Biceps femoris		
	61°C	80°C	100°C	61°C	80°C	100°C
Shears vs.[b]						
Juiciness	.05	−.13	−.07	−.09	−.17	−.07
Softness to tc	−.61	−.56	−.61	−.20	−.33	−.37
Softness to tp	−.72	−.81	−.83	−.27	−.52	−.67
Ease of fragmentation	−.62	−.84	−.82	−.16	−.65	−.76
Mealiness	....	−.62	−.68	....	−.50	−.65
Adhesion	−.62	−.79	−.83	−.22	−.65	−.76
Tenderness of ct	.17	−.10	−.29	−.22	.12	−.11
Weight loss	.35	.26	.41	.18	.28	.34
Cooking time	.09	.02	.12	−.10	.29	.06
Extensibilities vs.[c]						
Juiciness	.14	....	.03	−.13	....	−.03
Softness to tc	−.52	....	−.42	−.27	....	−.36
Softness to tp	−.63	....	−.70	−.16	....	−.66
Ease of fragmentation	−.59	....	−.74	−.31	....	−.71
Mealiness	....	....	−.66	....	....	−.68
Adhesion	−.61	....	−.74	−.39	....	−.66
Tenderness of ct	.14	....	−.16	.16	....	−.17
Shears	.83	....	.78	.06	....	.78
Weight loss	.26	....	.33	.40	....	.24
Cooking time	.00	....	.10	.09	....	.11

[a] Abbreviations: tc, tongue and cheek; tp, tooth pressure; ct, connective tissue.
[b] Coefficients below .16 not significant; between .16 and .21 significant at 5% level; above .21 significant at 1% level.
[c] Coefficients below .17 not significant; between .17 and .23 significant at 5% level; above .23 significant at 1% level.

of fragmentation of muscle fibers, and adhesion between muscle fibers have a common cause is not known, but it seems unlikely. Shear-force values do not distinguish among them and would be of little value in the search for possible independent causes.

Shear-force values are obtained easily and are used widely. It would be convenient if they could be obtained on one muscle by one method of cooking and the results applied to the tenderness of all the meat in a carcass. To test such a possibility on two muscles, the correlations were pooled within lots for all possible combinations of the two muscles and the three conditions of cooking (Table 3). Coefficients were nonsignificant when BF at 61°C was correlated with LD at each of the three temperatures. Significant positive coefficients were obtained when BF at 80 and 100°C was correlated with LD at each of the three temperatures, but they indicate that the variation in shear-force values in one muscle accounts for less than 36% of the variation in the other muscle. Thus, results obtained with one muscle would appear to be less than satisfactory for indicating the tenderness of another muscle in the same carcass. In these correlations between muscles, the very low coefficients for BF at 61°C indicate that something affecting shear-force values is present in BF at 61°C that is either not present in LD or not present in the same amount. If this were connective tissue alone, the higher coefficients obtained with BF at 80°C might have been expected, but still-higher ones would have been expected at 100°C. Thus, more than one factor seems to be involved in causing the differences in shear-force values between the two muscles.

The correlations in Table 3 also give information on the associations within a muscle. Within BF, positive coefficients, significant but very low, were obtained between 61°C and each of the other two

temperatures whereas the positive coefficient between 80 and 100°C was not only significant but high. Within LD, the positive coefficients among all the temperatures were also significant and high. The high coefficients suggest several conclusions: 1) Those animals with the highest shear-force values at one temperature tended to have the highest at the other temperature also. 2) The meat within these comparisons was affected by heat in much the same way. 3) The animals were ranked in about the same order for all of the temperatures within LD, but for only two temperatures (80 and 100°C) within BF. 4) Standardization of the experimental techniques must have been satisfactory. 5) The variation among animals needed to obtain these high coefficients must have been caused by differences prior to cooking. Such differences may have occurred during processing, during the life of the animals, or through heredity.

Variations in shear-force values have been noted from end to end of the muscle in steaks or chops (Weir, 1953; Mackey and Oliver, 1954; Paul and Bratzler, 1955; Ginger and Weir, 1958; Batcher and Dawson, 1960) and from side to side within a chop (Murphy and Carlin, 1961). In this study, obtaining cores from 4 positions within a steak permitted further investigation of this problem. Statistical treatment was by the outline of the analysis of variance in Table 1 for both muscles.

In LD, differences between core positions within a steak were significant for some lots but not for all. However, there is no evidence in Fig. 2 that the area nearer the vertebrae (cores one and two) was more tender than that farther from the vertebrae (cores three and four), as was reported in 9–10–11 rib roasts with panel scores (Cover, 1937) or in pork loin chops with shear-force values (Murphy and Carlin, 1961). In fact, the data in Fig. 2 indicate that the tenderest area, especially at 100°C, may have been around core three (farthest from the vertebrae and near the fat edge). In the analysis of variance (Table 1) temperature differences for LD were significant at the 0.1% level in all lots. Shear-force values of the cores in all positions increased as the temperature increased from 61 to 80°C. This change was responsible for the foregoing significance because there was little change between 80 and 100°C (Fig. 3). The interaction between temperatures and cores was not significant except in lot 4. This means, in general, that meat from all positions in LD reacted to heat in a similar manner.

In BF, however, differences between core positions were not significant in any lot. Temperature differences were significant in only three lots. But the interaction cores × temperatures was significant for all nine lots (0.1% for 7 lots, 1.0 and 5.0% for one lot each), and therefore the test of the main effects is not straightforward. This means that some areas of the steak reacted to heat in a manner different from the others. In Fig. 2, cores one and three usually sheared lowest of the four cores at 80 and 100°C, but at 61°C core three often had the highest shear-force value of the four cores. For cores two and four from the area of the muscle nearest the bone and for core one on the fat side of the muscle, shear-force values increased slightly be-

Table 3. Pooled correlations calculated within lots for all possible combinations of muscle and conditions of cooking. (Shear-force values).

		Pooled coefficients				
		Longissimus dorsi		Biceps femoris		
Variables		80°C	100°C	61°C	80°C	100°C
Longissimus dorsi	61°C	.81**	.81**	.16	.59**	.56**
	80°C		.91**	.14	.59**	.55**
	100°C			.11	.60**	.60**
Biceps femoris	61°C				.39**	.29**
	80°C					.86**

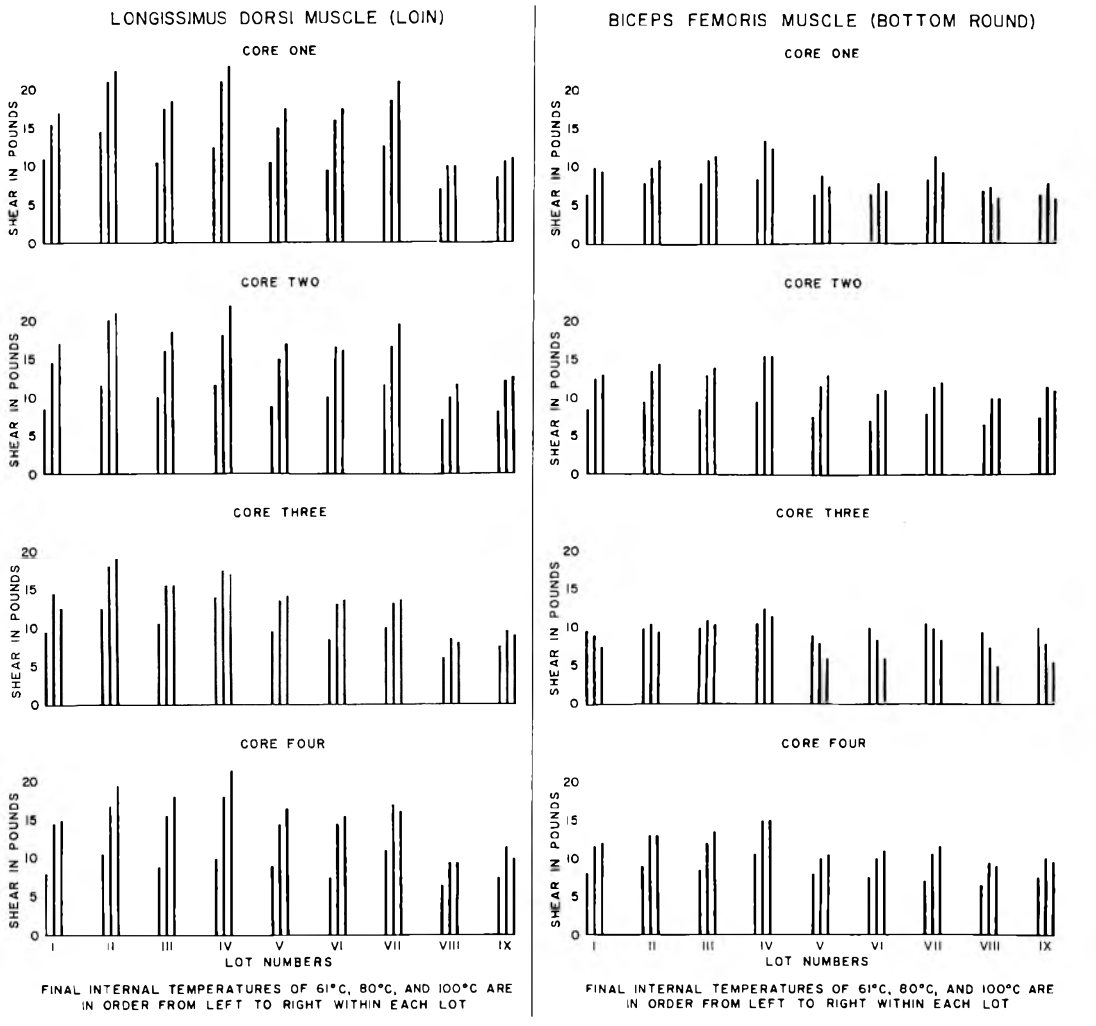

Fig. 3. Shear-force values averaged by core positions. Three temperatures within each core position.

tween 61 and 80°C but usually showed little further change between 80 and 100°C (Fig. 3). For core three, also on the fat side of the muscle, shear-force values often decreased with increase in final temperature, a complete reversal of the trend in cores two and four. Such variations contribute evidence that meat is not homogeneous. Sampling techniques must be planned to minimize this lack of homogeneity. Thus, using four cores from definite areas separated as widely as possible within a steak, and averaging them to get a composite value for the steak, tended to minimize this lack of homogeneity.

Extensibility of muscle fibers. In LD, the muscle fibers were much more extensible at 100 than at 61°C, a difference that was significant in all lots used (Fig. 4). In BF, the trend was in the same direction but differences between the two temperatures were significant only in lots 3, 7, and 9. The fibers were much more extensible in LD than in BF. This difference was significant at both temperatures for all lots used.

Interrelations of extensibilities with panel scores and with shear-force values were obtained within a muscle and condition of cooking by correlations pooled within lots (Table 2). In LD the values most closely related to extensibility at each temperature were the shear-force values—positively correlated. This indicated that the

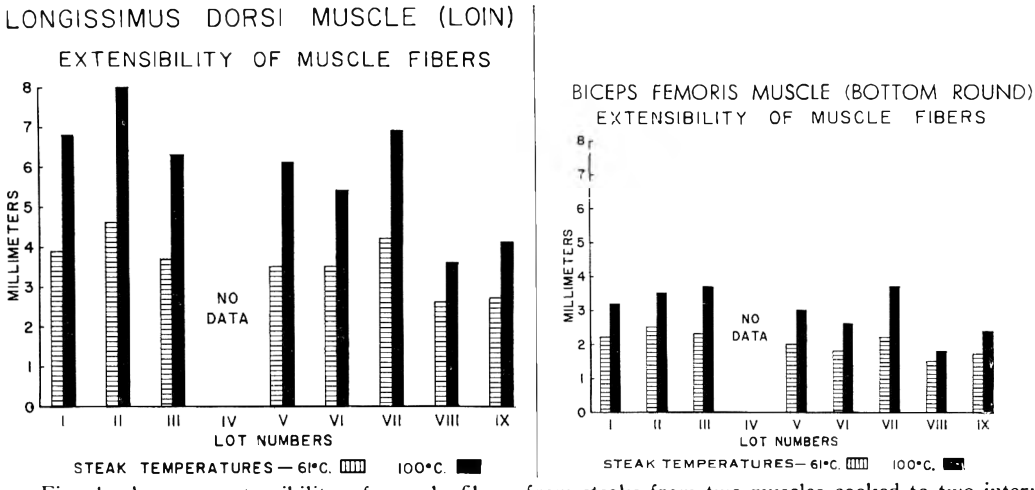

Fig. 4. Average extensibility of muscle fibers from steaks from two muscles cooked to two internal temperatures.

greater stretch was associated with the higher shear-force values (tougher meat). In LD at 100°C, where extensibility was most pronounced, none of the coefficients with scores was really high, but the highest were for ease of fragmentation, adhesion, softness to tooth pressure, and mealiness—all negatively correlated. In BF, extensibility was rather closely and positively related to shear-force values at 100 but not at 61°C. In BF the relation also was considerably closer at 100 than at 61°C between extensibility and the scores for ease of fragmentation, adhesion, and softness to tooth pressure—all negatively correlated. The relation between extensibility and panel scores for tenderness of connective tissue was low for both muscles at both temperatures. Thus, the data suggest that extensibility, like shear force, seems to be measuring mainly the characteristics of the muscle fiber.

Hostetler and Cover (1961) noted that fibers from some blended samples of BF at 61 and 100°C were too short for extensibility determinations. It was usually possible in the present study to get fibers from cores two and four (those nearest the bone) but often not possible from cores one and three at either of the internal temperatures. Evidently there is a real difference between the muscle fibers in this area close to the bone and those on the outer side of the BF muscle.

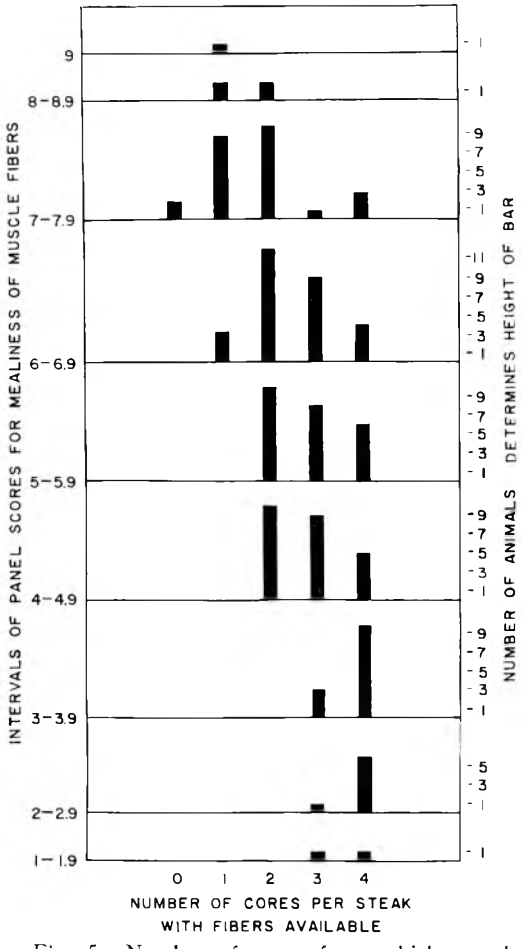

Fig. 5. Number of cores from which muscle fibers were available arranged according to panel scores for mealiness in the same steak. Biceps femoris at 100°C.

A possible relation may be indicated between muscle fiber availability and panel scores for mealiness in BF at 100°C (Cover *et al.*, 1962c). It was found that, if the score for mealiness was 3.7 (very slightly mealy) or below, muscle fibers from that steak were always available from at least three cores, but usually from four. On the other hand, if the mealiness score was 7.0 (mealy) or above, fibers were usually available from only two cores or fewer. Steaks with scores between 3.7 and 7.0 showed intermediate availabilities of fibers (Fig. 5). This seems to indicate that the quality detected by the panel as mealiness may be related to a physical characteristic of BF cooked to 100°C (very short muscle fibers). Mealiness scores at 61°C were very low because at that temperature any very short muscle fibers which were present were nearly always moist and soft instead of dry and hard—a requirement for the mealy sensation. Yet within the same animal the availabilities were rather similar at 61 and 100°C. Cover *et al.* (1962c) suggested a possible connection between mealiness and the cracks and breaks in the muscle fibers developed during aging of the meat reported by Paul *et al.* (1952). The very short muscle fibers reported here could have been cracked or broken before cooking. This possibility should be investigated.

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Reticulataxanthin and Tangeraxanthin, Two Carbonyl Carotenoids from Tangerine Peel

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SUMMARY

Reticulataxanthin and tangeraxanthin were obtained from tangerine peel; both occurred in the diol fraction on countercurrent distribution, and on chromatography were found on the column above zeaxanthin as violet-red and violet bands, respectively. Both contained a carbonyl group as a part of the conjugated-double-bond system. Reticulataxanthin was shown to be different from capsanthin, hydroxycanthaxanthin, and citraurin. Both of these carbonyl carotenoids apparently contained fewer than 40 carbon atoms, perhaps 34 or 35.

In earlier work at this laboratory, a pigment with a spectral absorption curve similar to that of capsanthin (Polgár and Zechmeister, 1944) was found in small amounts in the peels of Valencia (Curl and Bailey, 1956) and navel oranges (Curl and Bailey, 1961) and in considerably larger amounts in tangerine peel (Curl and Bailey, 1957). This pigment is responsible, at least in part, for the redder color of tangerine peel. In earlier papers, the pigment was referred to as capsanthin-like or hydroxycanthaxanthin-like. It was later found to be distinctly different from these pigments, so was named "reticulataxanthin" (Curl and Bailey, 1961). This pigment has now been further investigated, as well as a second unusual carotenoid, "tangeraxanthin," also obtained from tangerines.

EXPERIMENTAL

Three lots of fruit were obtained at a local market on December 12, December 20, and January 26. The weights of peel extracted were respectively 995, 1215, and 1714 g. In batches of around 500 g, the peels were blended for 1 min with 1000 ml of methanol, 1700 ml of water, and magnesium carbonate (1% by weight of the peels). The blends were worked up as previously described (Curl and Bailey, 1961), including saponification by potassium hydroxide in a one-phase system of ether-methanol. The saponified carotenoids were then dissolved in a mixture of hexane and 95% methanol, the layers well mixed, the lower layer separated, and the upper layer extracted twice more with 95% methanol.

The carotenoids in the combined lower layers were then further fractionated by countercurrent distribution in a 101-tube Craig apparatus, using a new solvent system (V, Table 1). The diol fraction, which included reticulataxanthin and tangeraxanthin, was a strong red color, and was separated visually as the upper layer transferred from tube 100 in transfers 140 to 199. The diol fraction was chromatographed on magnesia (Sea Sorb 43) (Curl, 1961). Spectral data (Table 2) were obtained in a Beckman DK-2 spectrophotometer.

Table 1. Solvent systems used in countercurrent distribution.

No.	Components	Relative volumes
I	hexane, 99% methanol [a]	1.8:1
II	benzene, hexane, 87% methanol	1:1:1.15
IV	hexane, 73.5% methanol	1:1
IVA	hexane, 75% methanol	1:1
V	hexane, acetone, 37.5% methanol	1:1.15:0.43

[a] Consists of 99% methanol and 1% water.

Several carbonyl carotenoid solutions were reduced by sodium borohydride in 95% ethanol (Curl, 1962). The rates of reduction by sodium borohydride of several carbonyl carotenoids were compared by the method of Critchley *et al.* (1958), using as solvents both absolute and 95% ethanol. Other carotenoid solutions were treated with hy-

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

Table 2. Spectral absorption maxima and N_{100} values[a] of various carotenoids.

Carotenoids	Spectral absorption maxima (mμ)	Solvent[b]	N_{100} value[c]
Reticulataxanthin	(507), 478, (457)[d]	B	67(II) : 64(IVA)
	495, 465	H	
	470	E	
Reticulataxanthol	486, 456, 431	B	48(II) ; 47(IV)
Reticulataxanthol methyl ether	465, 436, (412)	H	31 (I)
Tangeraxanthin	(520), 484, (463)	B	
Tangeraxanthol	494, 463, 437	B	56 (II)
Tangeraxanthol methyl ether	480, 450, 427	H	32 (I)
Tangeraxanthol dimethyl ether?	(480), 451, (428)	H	76 (I)
β-apo-2-carotenal	(493), 468	B	60 (I)
	480, 455	H	
β-apo-2-carotenol	460, 433, (410)	B	42 (I)
Capsanthin	(510), 484	B	45 (II) ; 23(IVA)
	498, 469[e]	H	
Capsanthol	487, 456, (430)	B	22 (II)
Dehydrocapsanthol methyl ether	495, 464, 439	B	39(I) ; 93(II)
Canthaxanthin	480	B	94(II) ; 34 (I)
	464	H	
Citraurin	497, 467[e]	B	

[a] N_{100} value is position of maximum per 100 transfers on countercurrent distribution.
[b] B, benzene; H, hexane; E, ethanol.
[c] Roman numerals designate solvent system (Table 1).
[d] Values in parentheses are for shoulders or inflections on spectral absorption curves.
[e] Values from literature.

drochloric acid in methanol (Curl and Bailey, 1954).

Solvent systems used in countercurrent distribution runs are given in Table 1.

RESULTS AND DISCUSSION

Reticulataxanthin. On countercurrent distribution with system II (Table 1), reticulataxanthin occurred in the diol fraction (Curl and Bailey, 1957). To obtain larger quantities of this substance (a minor carotenoid constituent), considerably larger quantities of material than usual were used, resulting in the formation of slowly separating emulsions. This was alleviated in part by use of a preliminary phasic separation between hexane and 95% methanol. Separations were much more rapid with a new solvent system (V, Table 1), which gives a separation similar to that with system II, except that, unlike in system II, 5,8-epoxides have appreciably higher N_{100} values than the corresponding 5,6-epoxides. This did not prove to be a handicap in the present study, where the object was primarily to separate reticulataxanthin, but does make system V of less value in a general carotenoid examination where system II is used to separate the polyol, diepoxide diol, monoepoxide diol, and diol fractions.

As reported earlier (Curl and Bailey, 1957), on chromatography of the diol fraction from tangerine peel, a strong violet-red band (reticulataxanthin) occurred above zeaxanthin, which was eluted from the column with 10–15% ethanol in hexane. In the carotenoids from the fruit obtained in December, a violet band (tangeraxanthin) occurred above reticulataxanthin, which was eluted by 15–25% ethanol in hexane. This band was absent from the carotenoids from the fruit obtained in late January, and it was not observed in earlier work (Curl and Bailey, 1957) on tangerine peels, in which the fruit was obtained on January 19.

The spectral absorption curve of reticulataxanthin (Curl and Bailey, 1957) was similar to that of capsanthin (Polgár and Zechmeister, 1944), but the principal maximum (for a crystalline preparation) was at 478 instead of 484 mμ (in benzene). Capsanthin, a monoketo diol, was prepared from red garden peppers (in which it is by far the most abundant carotenoid)

(Curl, 1962). On countercurrent distribution in solvent systems II and IVA, the N_{100} values of capsanthin were much lower than those of reticulataxanthin (Table 2). These results show definitely that the tangerine pigment was not capsanthin. Three minor carotenoid constituents of red peppers of incompletely known structure also had spectral absorption curves similar to that of capsanthin, but these also differed markedly from reticulataxanthin in N_{100} values.

Canthaxanthin (4,4′-diketo-β-carotene) had a principal spectral absorption maximum in benzene at 480 mμ, close to that of reticulataxanthin. The N_{100} value in system II was 94, much higher than that of the tangerine pigment. In an earlier report (Curl and Bailey, 1957), it was considered possible that the tangerine pigment was a hydroxycanthaxanthin. The spectral absorption curve in hexane of capsanthin (Polgár and Zechmeister, 1944) had two maxima, at 498 and 469 mμ. The tangerine pigment likewise had two maxima, at 495 and 465 mμ, in hexane. Canthaxanthin in hexane had a single maximum at 464 mμ, with no apparent inflection. This indicates that reticulataxanthin has a chromophore similar to capsanthin, but different from canthaxanthin, hence is quite unlikely to be a hydroxycanthaxanthin.

Zechmeister and Tuzson (1937) found an unusual red pigment in the peel of Sicilian oranges, for which the name citraurin was proposed. This pigment, $C_{30}H_{40}O_2$, was an aldehyde, 3-hydroxy-β-apo-2-carotenal (Fig. 2c). The spectral absorption maxima of citraurin were at 497 and 467 mμ in benzene, values somewhat lower than those of reticulataxanthin. Citraurin was not available for comparison, but β-apo-2-carotenal was. The spectral absorption maximum of this substance in benzene was at 468 mμ, with a minor inflection at 493 mμ, and in hexane there were two maxima at 480 and 455 mμ. The values of β-apo-2-carotenal were thus *ca.* 10 mμ shorter in wavelength than those of the tangerine pigment, hence the latter is obviously different from citraurin.

Solutions of carotenoids having a carbonyl group as a part of the conjugated-double-bond system, such as β-apo-2-carotenal, canthaxanthin, capsanthin, and capsorubin, have a considerably redder color in ethanol than in the same concentration in hexane or petroleum ether, and the spectral absorption maximum in ethanol is about 9 mμ higher in wavelength, whereas solutions of non-carbonyl carotenoids have similar color and absorption maxima in ethanol and in hexane. The solution of reticulataxanthin was also considerably redder in ethanol than in petroleum ether, and the spectral absorption maximum was at 470 mμ, whereas in a solution of the same material in petroleum ether it was at 463 mμ. This indicates that the tangerine pigment has a carbonyl group as a part of the conjugated-double-bond system.

Capsanthin has a conjugated-double-bond system with a keto group at one end, with 10 carbon-to-carbon double bonds, the last one being in the 5,6-position of the β-ionone ring, as in β-carotene. The lower wavelength of the spectral absorption maxima of reticulataxanthin might indicate that no double bond is present in the 5,6-position. Two possibilities are that the double bond has been replaced by a 5,6-epoxide group, or that it has been shifted to the 4,5-position, as in α-carotene; both of these configurations are well known. The spectral absorption maximum of capsanthin-5,6-epoxide (Curl, 1962) was the same as that of reticulataxanthin (478 mμ in benzene).

It was found that the addition of one drop of hydrochloric acid to a solution of reticulataxanthin in *ca.* 4 ml of ethanol resulted in a decrease in wavelength of the spectral absorption maximum of only 1 mμ, whereas with a 5,6-epoxide the maxima are decreased *ca.* 20 mμ. This shows that the tangerine pigment is not a 5,6-epoxide.

In another experiment, to test for the presence of an allylic hydroxyl group, reticulataxanthin was dissolved in methanol containing hydrochloric acid, and the solution neutralized after standing for 10 min. The product was used in a countercurrent distribution run with solvent system II. The N_{100} value was found to be 68, a change of only 1. This indicates the absence of an allylic hydroxyl group as in an α-ionone ring with a hydroxyl group in the 3-posi-

tion. With lutein, which has such a configuration, the allylic hydroxyl group is methylated with a resulting considerable increase in N_{100} value in system I from 10 to 38 (Curl, 1956) (Fig. 1). Petracek

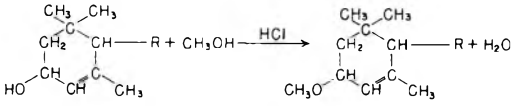
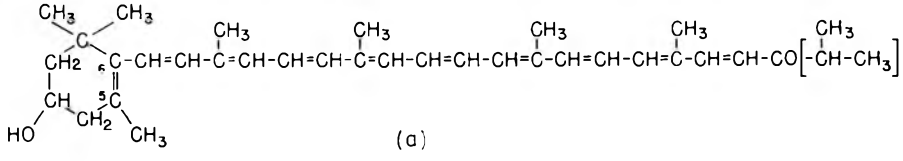


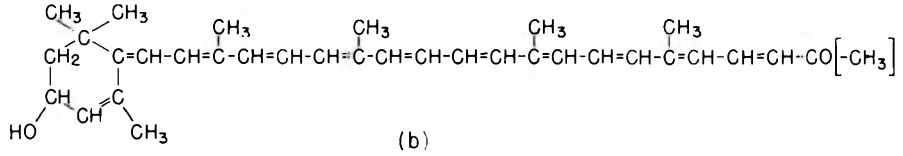
Fig. 1. Conversion of an allylic hydroxyl group, as in lutein, to the methyl ether.

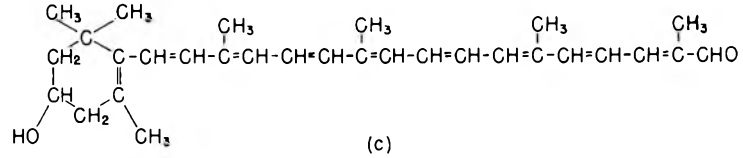
and Zechmeister (1956) showed that certain allylic hydroxyl groups in carotenoids were readily methylated by acid methanol. This methylation has been shown to occur in secondary allylic alcohol groups on rings like the α-ionone (Fig. 1); the behavior of allylic primary or tertiary alcohols, or of secondary alcohols in open chains, has apparently not been reported.

These results indicate that the tangerine pigment is distinctly different from capsanthin, citraurin, and canthaxanthin. It appears that it is a previously undescribed pigment, for which the name "reticulataxanthin" is proposed, since the principal source found for this pigment so far is in the peel of the tangerine *Citrus reticulata*. This pigment is mainly responsible for the redder color of tangerine peel than of orange peel.

Crystallization of reticulataxanthin. Reticulataxanthin was found to be readily soluble in benzene and methanol, and rather sparingly soluble in hexane. It was more difficult to crystallize than other carotenoids, such as lutein and cryptoxanthin, apparently because of its greater solubility, which may be related to a considerable difference between the two ends of the molecule. It was obtained in crystalline form by dis-







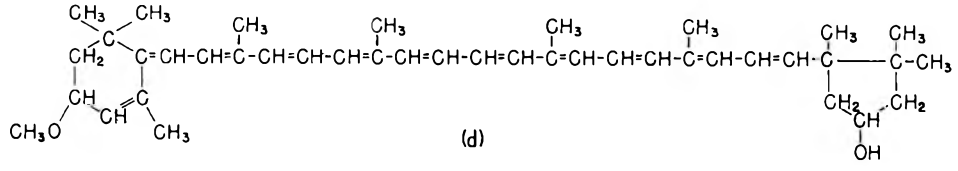


Fig. 2. Tentative structural formulas of: (a) reticulataxanthin; (b) tangeraxanthin; (c) citraurin (3-hydroxy-β-apo-2-carotenal) (definite); (d) dehydrocapsanthol methyl ether.

solving a quantity of about 8 mg in 2 ml of benzene, transferring to a ½-oz bottle, and filling the bottle carefully by layering on hexane. The bottle was tightly stoppered and allowed to stand on its side overnight in the refrigerator. The crystals were small rosettes adhering to the wall of the bottle; a relatively large amount of apparently colorless material was also present, adhering to the glass also. This colorless material was not removed by recrystallization. Because of this contamination the melting point and elemental analysis were not determined.

Preparation of reticulataxanthol. To a solution of crystalline reticulataxanthin in 95% ethanol was added some sodium borohydride, and the mixture was allowed to stand a few hours in the refrigerator. The solution became much paler in color. The recovered carotenoid had spectral absorption maxima in benzene at 486, 456, and 431 mμ, values only slightly lower than those obtained with crystalline lutein; the shape of the curve closely resembled that of lutein. It was obtained in crystalline form in very small amount by a procedure similar to that used with reticulataxanthin. The close agreement of the spectral absorption curve with that of lutein indicates the presence of a double bond in the 5,6-position. Reticulataxanthin and capsanthin apparently have the same conjugated-double-bond system. The difference in the spectral absorption maxima of these substances is probably due to the different groups in the molecule beyond the carbonyl group. Karrer and Jucker (1950) state: carbonyl groups conjugated with the system of conjugated double bonds have a pronounced effect on the absorption spectrum, and produce bathochromic displacements, the magnitude of which varies from case to case.

The N_{100} value of the reduced compound (reticulataxanthol) in system II was 48, compared with 67 for reticulataxanthin. The resulting compound, apparently a diol, had an N_{100} value much below that of zeaxanthin (69). This may be accounted for by the presence of somewhat fewer than 40 carbon atoms in reticulataxanthin.

The rate of reduction of reticulataxanthin by means of sodium borohydride in ethanol (both absolute and 95%) was compared with the rates of β-apo-2-carotenal, canthaxanthin, capsanthin and capsorubin (the latter two were obtained from red bell peppers) by the method of Critchley *et al.* (1958) for differentiating conjugated aldehydes and ketones. The results are given in Table 3. Critchley *et al.* obtained complete reduction (in alcohol) in 10 min or less for a number of conjugated aldehydes (β-apo-2-carotenal was 90% reduced in 10 min), the time for canthaxanthin (4,4′-diketo-β-carotene) was 60 min, and for several other conjugated ketones 60 to 100 min. In the present work, in absolute ethanol reticulataxanthin was practically completely reduced in 30 min, a value intermediate between the values obtained for β-apo-2-carotenal and canthaxanthin, while capsorubin and capsanthin were quite incompletely reduced in 120 min. From the results of Critchley *et al.*, it appears that an increase in the size of the molecule may result in a lower rate of reduction. Hence reticulataxanthin, which has one more conjugated double bond than β-apo-2-carotenal, and hence at least 2 more carbon atoms, may be a conjugated aldehyde.

Treatment of reticulataxanthol with hydrochloric acid-methanol. A solution of reticulataxanthol in hydrochloric acid-methanol became an opaque brown color. After standing 2 min, an excess of potassium hydroxide in methanol was added. A countercurrent distribution run using solvent system I was carried out on the recovered product. Three maxima were obtained, with N_{100} values of 78 (48%), 31 (45%), and 9 (6%); these values respectively correspond to a monoether or diether (?), a monol monoether, and a diol. The fraction

Table 3. Time for practically complete reduction of several carbonyl carotenoids by $NaBH_4$ in ethanol.

Carotenoid	Time (min) in ethanol solution	
	95%	100%
Reticulataxanthin	10	30
β-apo-2-carotenal	3	10
Canthaxanthin	16	85
Capsanthin	35	120+
Capsorubin	55	120+

with N_{100} value of 31 had spectral absorption maxima in hexane at 465 and 436, with a shoulder at 412 mμ; this was apparently a monol monoether with the methoxyl group on the carbon atom that was a carbonyl in reticulataxanthin, since the original hydroxyl group in reticulataxanthin was found not to be allylic. The spectral absorption maxima of this fraction are about 8 mμ lower in wavelength than those of reticulataxanthol; this may indicate conversion to *cis* or *dicis* isomers; the hydrochloric acid-methanol treatment usually results in considerable *trans-cis* isomerization.

The maximum with N_{100} of 78 was unusually wide, with shoulders at *ca.* 69 and 92 (hydrocarbons). Since this fraction was obviously a mixture of at least 3 components, an attempt was made to rechromatograph it. However, nearly all of the color remained at the top of the column (some even remained in the sodium sulfate layer) and showed no signs of being eluted by 1% ethanol in hexane, which elutes most monols readily; only about 5% of the pigment was eluted, with absorption maxima in hexane at 464 and 436 mμ, close to those of the N_{100} 31 fraction. Apparently a major part of the reticulataxanthol had been converted to an unstable reactive substance, possibly a polymer; the formation of the brown color on the hydrochloric acid treatment was unusual.

Fig. 2a shows a tentative formula for reticulataxanthin, based on the above results. The portion of the molecule in brackets is uncertain; it may be simply a hydrogen atom.

Tangeraxanthin. The violet band occurring on the column above reticulataxanthin had a spectral absorption maximum of 484 mμ (in benzene), with pronounced inflections at 520 and 463 and a *cis*-peak at 378 mμ. These values are similar to maxima observed in capsorubin (520, 486, (460), and 380), a diketo diol occurring in red peppers, though the shape of the curve was quite different. This band amounted to less than 10% as much as the reticulataxanthin.

Reduction of tangeraxanthin. On sodium borohydride reduction, the solution changed from a rather strong pink to yellow. The spectral absorption maxima in benzene were now at 494, 463, and 437 mμ; the change indicates the presence of one carbonyl group in the conjugated-double-bond system. The above maxima are in good agreement with those of dehydrocapsanthol methyl ether (Table 2), tentative structural formula, Fig. 2d (Curl, 1962), formed by the action of hydrochloric acid in methanol on capsanthol. The N_{100} value of the reduction product in system II was 56, a value somewhat higher than that of reticulataxanthol (48), which may indicate the presence of 2 hydroxyl groups in a larger molecule, or else one of the hydroxyl groups is in a somewhat less exposed position.

Treatment of tangeraxanthol with hydrochloric acid-methanol. The solution in methanol was allowed to stand 2 min after addition of the hydrochloric acid. A countercurrent distribution run in system I was carried out on the reaction product. The main fraction (63%) had an N_{100} of 32 (a monol monoether ?), with minor fractions at 9 (5%) (diol), 63 (11%), and 76 (22%). The spectral absorption maxima of the main product (in hexane) were at 480, 450, and 427 mμ, indicating no change in the conjugated-double-bond system. The maxima of the N_{100} 63 and 76 fractions were similar to those of the N_{100} 32 fraction, indicating that no further conjugated double bonds were formed (one of the minor fractions was probably a diether).

A tentative formula for tangeraxanthin is given in Fig. 2b. The structure of the part of the molecule in brackets is uncertain; the chain might possibly end with a CHO group. The hydroxyl group in the ring, being allylic, would be expected to be methylated readily (as in lutein), the hydroxyl group formed by reduction of the carbonyl group perhaps less readily.

β-apo-2-carotenal. For comparative purposes, β-apo-2-carotenal was reduced with sodium borohydride. The spectral absorption maxima of the product in benzene were at 460, 433, and (410) mμ. The N_{100} value in system I was 42 (β-apo-2-carotenal in this system was 60). The N_{100} value of cryptoxanthin, a typical C_{40} monol, was 56–57. The lower value for β-apo-2-carotenol was caused primarily by the much smaller molecule (C_{30}), though the different

position of the hydroxyl would have some effect.

Reaction of β-apo-2-carotenol with hydrochloric acid in methanol-ether. After treatment for 5 min with hydrochloric acid in methanol-ether (1 : 9 : 5, by volume), a countercurrent distribution run was carried out with system I. Two major maxima and one minor were obtained, with N_{100} values of 93 (63%), 79 (32%), and 42 (5%), corresponding respectively to hydrocarbons, monoethers, and monols. The spectral absorption maxima of the first fraction were at much shorter wavelengths than those of β-apo-2-carotenol, indicating a loss of 1 or 2 of the conjugated double bonds. The fraction with N_{100} of 79 had spectral absorption maxima in hexane at 445 and 421, with a shoulder at 400 mμ; these values are several mμ lower than those of the starting material, which may be accounted for by the formation of *cis*-isomers. It appears that in β-apo-2-carotenol, the primary alcohol group (which is allylic) formed a methyl ether in acidic methanol, but the major part of the material was converted to a hydrocarbon or hydrocarbons of unknown structure, with a shorter conjugated-double-bond system than in β-apo-2-carotenol. Possibly a polymer was formed.

In all three experiments in which reduced carbonyl carotenoids were treated with hydrochloric acid in methanol, a considerable part of the products consisted of substances with N_{100} values considerably above those of the monomethyl ethers. In the case of tangeraxanthol, which apparently has two allylic hydroxyl groups, this consisted in part of a diether, but in the other two experiments, major parts of the product consisted of substances containing fewer conjugated double bonds than the starting material. This phenomenon had not been observed in numerous other carotenoids tested for allylic hydroxyl groups, except for compounds containing 5,6-epoxide groups. Apparently a dehydration of the alcohol group at or near the end of the side chain occurred, followed by other reactions, possibly involving polymerization. In some cases at least, it appears that allylic alcohol groups at or near the end of the side chain are not methylated as cleanly as when attached to carbon atoms in a six-carbon ring. The similarity in behavior of reticulataxanthol and β-apo-2-carotenol on treatment with hydrochloric acid-methanol may indicate that the chain ends with a CH_2OH group in both compounds, and that, in reticulataxanthin, the chain may end in a CHO. The different behavior of tangeraxanthol may be due to the presence of a —CHOH—R group at the end of the chain, with R being an alkyl group such as methyl; tangeraxanthin would then be a ketone.

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

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New Factors Involved in the Denaturation of Frozen Cod Muscle Protein [a]

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(Manuscript received June 11, 1962)

SUMMARY

Earlier work has shown that when the concentration of tissue salts in liquid solution in frozen cod is increased by freezing at a certain rate, the rate of denaturation of the proteins of the flesh is enhanced during subsequent cold storage. The present work is concerned with conditions under which the proportion of ice and concentration of tissue salts decreases, and with the subsequent behavior of the muscle in cold storage.

Cod were frozen before the onset of rigor mortis, vacuum-dried at −29°, embedded in paraffin and histologically sectioned. The ice crystals showed up as holes. Measurements of the areas of muscle and ice in photographic enlargements of the sections showed that the pre-rigor frozen fish formed relatively less ice than post-rigor frozen fish under the same conditions. It was further shown that the pre-rigor fish then underwent less denaturation after cold storage than did the controls.

Storage time/denaturation curves at narrowly-spaced temperatures near the melting point showed that the rate of denaturation did not continue to increase as the melting point was approached. Above a certain temperature the rate began to decrease, and this again appeared to be due to the decrease in the proportion of ice, increase in free water, and consequent dilution of tissue salts.

The effect of decreasing the amount of ice in the tissue by the use of glycerol, and of the abolition of ice altogether by ultra-rapid freezing, is described.

When cod are frozen, the muscle proteins gradually deteriorate. The appearance of the muscle becomes dull and rough, much "drip" exudes on thawing out, and the product becomes tough and fibrous to eat. The extent to which this undesirable process ("denaturation") has occurred in a sample of muscle can be assessed by measuring the solubility of the proteins in 5% NaCl after homogenization (Dyer, 1951) or, with greater accuracy, by measuring the resistance of the individual cells to mechanical breakdown (Love and Mackay, 1962).

While, in general, the rate of denaturation is governed solely by the sub-zero storage temperature, becoming progressively reduced as the temperature is reduced (Love, 1962), one particular condition of freezing can increase the rate of denaturation considerably, even at low temperatures (Love, 1958).

At this critical freezing rate, when the muscle cools from 0 to −5°C in about 60 min, each cell contains just one ice crystal, which grows up the center. Fig. 1 shows a longitudinal section of such muscle. It has been concluded (Love, 1958) that the cell minerals and small organic molecules such as sugars become more concentrated at one end of each cell in these circumstances than in any other. It seemed that denaturation occurred only in the presence of concentrated salts, etc., *in liquid solution*, and that therefore the denaturation would cease altogether at very low temperatures where the last trace of liquid had disappeared.

[a] The work described was carried out as part of the program of the Department of Scientific and Industrial Research.

Fig. 1. Longitudinal section of fish frozen at the critical speed, about 60 min. Light areas represent ice. Each cell contains just one column of ice growing up the center. On left is the end of one ice crystal: it is here that salts are believed to accumulate and cause accelerated denaturation. Distance left to right represents about 1 mm of original tissue.

The present paper describes attempts to reverse the special case by decreasing the quantity of ice formed at any particular temperature, and hence lowering the concentration of tissue salts with the object of decreasing the amount of protein denaturation.

MATERIALS AND METHODS

The cod (*Gadus callarias* L.) used throughout the work were caught by trawl net a few miles east of Aberdeen, gutted immediately, and packed in ice. They were worked up within 24 hr of being caught. The fish from which the pre-rigor muscle was taken were rested alive in large sea-water aquaria maintained at 9°C. They were fed on the chopped muscle of squid (*Loligo forbesii* Steenstrup).

Since cod muscle is markedly affected by a number of biological factors, the sampling techniques of Ironside and Love (1958) were followed to eliminate them, ensuring a raw material with but slight variation in composition and properties.

The proportion of ice in the frozen tissue was calculated by preparing histological cross-sections 10 μ thick, essentially by the method of Koonz and Ramsbottom (1939), and staining with hematoxylin and eosin. Images of the sections magnified 125 times were projected onto photosensitive paper, a graticule of about 1100 squares being incorporated into the final picture. The proportions of ice to tissue were calculated by counting the squares, using an electromagnetic counter. Thirty photographs were scanned for each set of freezing conditions, representing an area of 75 sq mm of actual fish measured (about 3000 cells). A magnification of 500 times was used in the rapidly-frozen specimens because of the much smaller size of the ice crystals, so that the area of actual fish scanned was correspondingly smaller. Because a certain amount of shrinkage occurred during preparation of the sections, the figures for the proportion of ice cannot be related to those obtained by other methods, e.g., calorimetry. The method was felt to be suitable for comparative purposes, however, since the results were satisfactorily reproducible and the technique was simple to carry out.

Soluble protein determinations were carried out according to the method of Ironside and Love (1958) with a homogenizer speed of 8750 rpm.

The cell fragility method was used as described by Love and Mackay (1962).

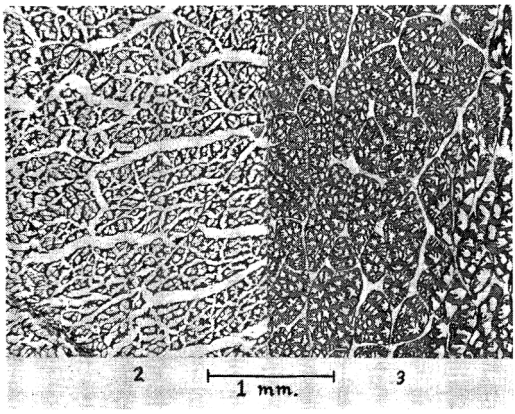
For storage at temperatures close to the freezing point, the fish were kept in cabinets in a room maintained 5–10°C below their working temperature. The cabinets were fitted with a sensitive temperature control of the contact thermometer type which controlled the cutting in or out of a heater. In addition to an insulated outer door, the cabinets had an inner glass door, through which could be observed the calibrating thermometer, mercury in glass, which had a scale ranging from 0° to −6°C, divided into hundredths, and which was itself calibrated at the National Physical Laboratory. The bulb was immersed in a beaker containing about ½ L of 40% glycerol, to smooth out minor variations in the temperature. The maximum variation in the temperature of the internal air, which was circulated with a fan, was ±0.25°C, but the fluctuations in a fish sample were much smaller than this.

RESULTS AND DISCUSSION

Pre-rigor muscle. Muscle rapidly frozen before the onset of rigor mortis ("pre-rigor muscle") was found to contain 39.8% of ice (standard error 3.08), whereas muscle frozen under the same conditions after the resolution of rigor contained 60.0% (standard error 3.24).

The microscopic appearance of the two types of muscle can be contrasted in Figs. 2 and 3 (the graticule has not been included here), and the smaller proportion of ice in the pre-rigor muscle (right-hand picture) is readily seen. Such ice is more lobular or rosette-shaped than that formed in post-rigor muscle, a property discussed elsewhere (Love and Haraldsson, 1961).

When fillets had been frozen more slowly, cooling from 0° to −5°C in more than 100



Figs. 2 and 3. Cross sections of (2) post-rigor and (3) pre-rigor cod muscle, each frozen in about 5 min. Note the rosette shapes of the ice crystals in Fig. 3, and the smaller proportion of ice.

minutes, there was a smaller, though still significant, difference in the amounts of ice formed in pre- and post-rigor muscle, respectively 55.2% (standard error 1.2) and 62.4% (0.74) of the total. The reason for the smaller differences in slowly-frozen fish is not known, but possibly the longer freezing time allows some of the rigor changes to take place, so that there is less physiological difference between the two types of muscle.

When pre- and post-rigor cod fillets were frozen and cold-stored, it was found that the pre-rigor muscle suffered less denaturation than post-rigor.

The pre-rigor fillets were obtained from cod that had been rested for 4–6 days after capture. The controls (post-rigor) were of similar size and caught in the same vicinity by another ship, gutted at once, and stowed in ice for 5 days before the experiment. They were filleted just before freezing.

The fillets were frozen at 6 different speeds by stacking 6 deep in an all-aluminum welded box 2 mm thick, the floor of which was then cooled to −78°. The technique has been described (Love and Haraldsson, 1961). In each of the 6 layers were 5 pre-rigor fillets, 2 of which contained thermocouples in the middle of the thickest part of the muscle, and 5 post-rigor fillets treated similarly. A wide range of freezing times was obtained in this way.

When all the fillets had reached −60°C, they were separated, wrapped individually in aluminum foil to prevent drying, and stored 56 weeks at −29°. They were then thawed out overnight at 5°, and the denaturation was measured by the soluble-protein method.

The results in Fig. 4 show that the pre-rigor fillets were less denatured than their post-rigor counterparts in all cases. Each point in the figure represents the mean value of duplicate determinations on the mixed sample from 5 fillets. The reason why the post-rigor fillets froze rather more slowly than the corresponding pre-rigor fillets has been discussed already (Love and Haraldsson, 1961).

Pre-rigor muscle differs in many respects from post-rigor, so there are a variety of possible reasons why the pre-rigor fillets became less denatured than the post-rigor. However, since these results are in line with the rest of the paper, the difference in the amount of ice formed seems to be a likely cause.

Behavior at temperatures near the melting point. Frozen muscle just below its freezing point contains a relatively large proportion of unfrozen water (Moran, 1934; Riedel, 1956), which decreases sharply with a small temperature drop. Thus, according to Moran (1934), 36.5% of the water of beef muscle was unfrozen at −2.5°C, decreasing to 24.4% at −5°C. It seemed possible therefore that the amount of ice in frozen fish could be decreased to a point where the tissue salts were insufficiently concentrated to cause denaturation.

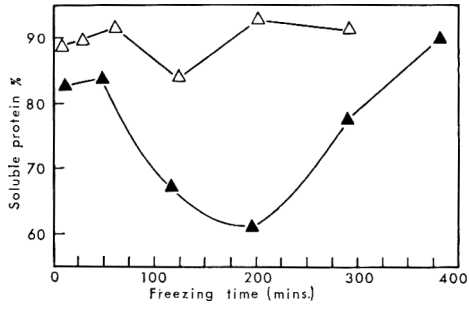


Fig. 4. Soluble protein of (△) pre-rigor and (▲) post-rigor cod muscle frozen at various speeds and then stored for a year at −29°C. Freezing time = time for center of thickest part of fillet to cool from 0° to −5°C.

Foil-wrapped pieces of post-rigor cod fillet were frozen at −29°C, then placed in cold cabinets at all temperatures from −3.5° to −0.5°C, inclusive, at half-degree intervals. Ten pieces, each from a different fish, were removed after various periods for measurement of denaturation by the cell fragility method (Love and Mackay, 1962), after thawing (in about 2 hr) in the laboratory, and the results averaged.

It has already been shown (Love, 1962) that at temperatures up to −4°C there is a steady increase in the rate of denaturation with increasing temperature. The present results showed that the increase continued from −3.5° up to −1.5°C, where the rate of reaction was maximum. It can be seen from Fig. 5 that the rate then decreased at −1.0° and decreased further at −0.5°C, although some ice was still present in the samples. The experiments were repeated in entirety, with closely similar results, the experimental points being in many cases identical with the corresponding previous points.

The curves in Fig. 5 have all been drawn through the same point of origin ($E_{1/2cm} = 0.914$), which is the starting point averaged over many experiments (Love, 1962).

The clear discrimination between the curves is remarkable in view of the very small temperature difference: it was made possible only by the high accuracy of the new "cell fragility method."

The striking effect of storing under identical conditions but without ice is shown as a horizontal line in the upper part of Fig. 5. It was found possible to keep unfrozen pieces of fillet, wrapped together in groups of 10, in the cabinet at −1.5°C without their freezing. All bundles stayed supercooled except one, which froze spontaneously and was discarded. The denaturation clearly depends on the presence of ice.

Fig. 6 shows, again, the curve of frozen fish at −1.5°C, but this time in relation to those at −2° and −2.5°C. Because of a rather larger scatter in the latter curves, it was not possible to draw them through a common origin, but the clear discrimination between curves can again be seen. The temperature of −1.5°C is therefore established

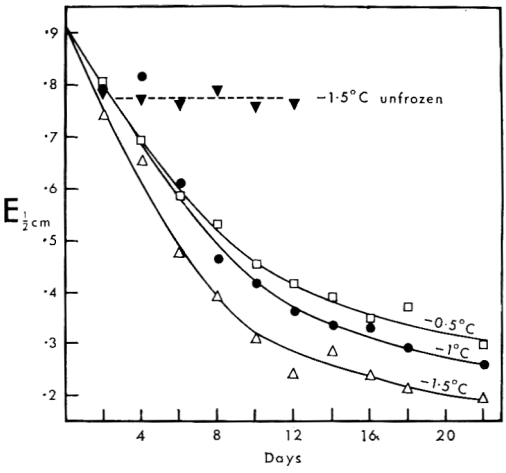


Fig. 5. Toughness development at storage temperatures of −1.5°C and above. A reduction in $E_{1/2cm}$ means an increase in toughness. Note that there is no toughness development in the supercooled samples (▼) where no ice is present. (After Love and Elerian, unpublished).

as the temperature of maximum denaturation in the cod. Fig. 5 provides a rare example of increased temperature resulting in decreased reaction rate.

The use of glycerol. It is now well known that the addition of glycerol to a suspension of spermatozoa (Polge *et al.*, 1949) and blood cells (Smith, 1950) greatly reduces the mortality or hemolysis caused by freezing. The glycerol was thought by Lovelock (1954) to act by lowering the concentration of cell salts in the frozen material, though Sloviter (1962) believes that its effectiveness rests on its ability to lessen the mechanical destruction usually wrought during ice crystal formation. In the present work the effect of glycerol on the denaturation of cod muscle was investigated because the underlying property of this substance, on which the above two theories are based, is that it can attach a considerable amount of water to itself, rendering it unavailable to form ice. The bound water can, however, still act as a solvent for cell salts, so that their concentration is kept low.

The anterior halves of a number of cod fillets were chopped into 5 pieces of equal length (about 4 cm). These were soaked in 15% v/v glycerol for 4 hr at 0°C, individually wrapped in aluminum foil to prevent drying, and frozen along with similar

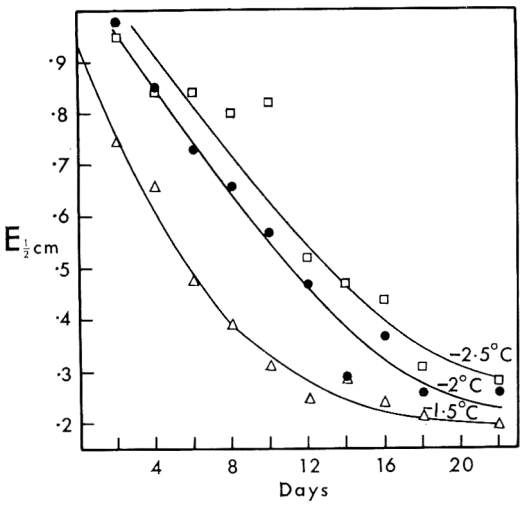


Fig. 6. Toughness development at storage temperatures of −1.5°C and below. (After Love and Elerian, unpublished).

but untreated pieces at −29°C. They were placed the following day in a room at −14°C for storage. Every two weeks, two pieces each of treated and untreated muscle were removed and thawed (in about 2 hr) in the laboratory. Muscle tissue (myotomes) uncontaminated by connective tissue (myocommata) was dissected out according to the method of Ironside and Love (1958), and mixed after cutting up with scissors. Two portions, of about 1 g each, were used for soluble protein determination.

The results obtained so far are illustrated in Fig. 7. Rate of denaturation was markedly smaller in the glycerol-treated material than in the controls, showing once more that the quantity of ice present is probably the governing factor. It is perhaps worthwhile recording that this concentration of glycerol imparted very little taste to the fish.

Further experiments, partially completed, have shown that 10% glycerol is also effective, but that 5% glycerol has little, if any, protective action. This observation agrees with that of Polge *et al.* (1949), who reported that the protective action of glycerol on frozen spermatozoa decreased when the concentration was reduced below 10%.

In some recent work, Tamoto *et al.* (1961) reported that mono- and disaccharides inhibited protein denaturation in cold-stored Alaska pollack, but that polysaccharides had no effect. The action here is presumably the same as in glycerol.

Vitrification. The concentration of tissue-salt molecules by freezing involves their being moved along the cell for a short distance, depending on the length of each ice crystal. It follows that if the muscle could be frozen without the formation of any discrete bodies of ice, then the tissue salts would not be locally concentrated, and there would be at least a chance of reducing the protein denaturation, even of eliminating it. An indication of this kind was shown in fish at −1.5°C, where denaturation occurred only if ice was present (Fig. 5). Luyet and Gehenio (1940) froze protein colloids without ice ("vitrification") by submerging very thin layers in liquid air, noting that crystals did form if the preparation was allowed to warm up.

In this laboratory, investigation showed that small pieces of cod muscle dropped into a large volume of iso-pentane cooled to −160.5°C formed no ice crystals, histological sections being indistinguishable under the microscope from those of fresh unfrozen fish. The advantage of using an inert liquid such as pentane or iso-pentane cooled in liquid air, instead of liquid air itself, was that no film of insulating gas formed round the specimen, so that cooling was more rapid. The technique is widely used.

Twenty samples of about 1 g of cod muscle, freed of connective tissue, were weighed. Ten of them were then placed in small stoppered glass tubes and frozen by placing in a vacuum jar with solid carbon dioxide (−78°C). These were to act as

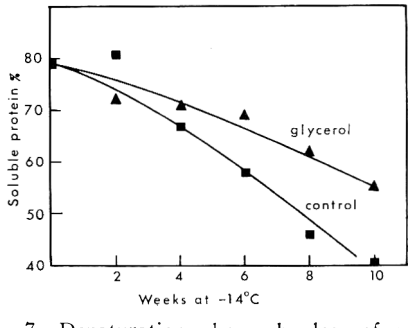


Fig. 7. Denaturation, shown by loss of protein solubility, occurring in cod muscle samples stored at −14° with and without a preliminary soak for 4 hours in 15% glycerol. (After Love and Elerian, unpublished).

controls, freezing taking place in 2–3 min, with only small ice crystals forming. The remaining ten were each chopped into 8–10 pieces of roughly equal size. These were dropped, one at a time, into about 100 ml of iso-pentane at −160.5°C. The iso-pentane, in a metal container, was surrounded with liquid air until it started to solidify, at which point the little pieces of fish were dropped in, freezing in less than a second. The frozen fragments were then removed and placed together in small stoppered tubes, like the controls, and all samples were placed in a room at −14°C for storage. Every two weeks, two vitrified and two control samples were removed and thawed in the laboratory (18°C) in about 10 min, and the denaturation was assessed by the soluble-protein technique.

The vitrified samples so far (up to 10 weeks at −14°C) have at each storage period denatured appreciably less than those in the "standard curve" obtained previously with many samples (Love and Ironside, 1958). Unexpectedly, however, the present controls behaved like the vitrified samples, and did not differ significantly from them in protein solubility. Perhaps the rather small ice crystals in the controls discouraged denaturation in the same way as did vitrification of the experimental samples, or maybe some ice crystals formed in the vitrified muscle after warming to −14°C. These possibilities are being investigated. Meanwhile the findings of this experiment are inconclusive.

The conclusion to be derived from all the results is that denaturation can definitely be slowed if the quantity of ice present at a given temperature is reduced. Since reduction in the amount of ice reduces the concentration of tissue salts, the results also support the contention (Love, 1958) that denaturation is caused, directly or indirectly, by the action of concentrated tissue salts on the proteins.

It seems clear that the best way to cold-store fish is still to keep them at as low a temperature as possible, ideally −30°C or below. This and the complete prevention of surface desiccation are the two most important factors.

Where a low storage temperature is not available, however, it is possible that storage life could be usefully extended by application of some of the more marginal factors described in this paper. The use of pre-rigor fish is advantageous, although they must not be filleted before freezing (Love, in press), and the color of the flesh is darker because of undrained blood. Whether the results of the application of glycerol or other sugars would justify their cost is perhaps doubtful, and it would be feasible only where high storage temperatures are to be used.

Such conditions have in fact been recently proposed, the intention being to slow the bacterial putrefaction of fish during long rail journeys (Golovkin and Pershina, 1961). In some large-scale experiments, those authors froze cod to low temperatures, then stored them 1–2 weeks at −1 to −2.5°C. The product was rated favorably as regards putrefaction, compared with iced fish, but the authors noted the reduced capacity of the frozen tissue to retain moisture after thawing, this being another manifestation of denaturation. Glycerol would have brought about improvement here. The present work also shows that the fish would have been improved by avoiding the temperature of −1.5°C. They could with benefit have been brought to the thawing temperature, near 0.4°C, or else kept below −3°C.

ACKNOWLEDGMENT

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Inhibition and Promotion of Post-Mortem Lipid Hydrolysis in the Flesh of Fish

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SUMMARY

In the flesh of certain species of fish, autolytic hydrolysis of some lipids may be totally inhibited. Examples are the hydrolysis of triglycerides in herring and of phospholipids in dogfish. In other cases an initially slow rate of hydrolysis of phospholipids may be followed by a faster rate. Such initial partial inhibition is exhibited, for example, in cod flesh maintained at 0°C. This initial "lag" may be abolished by freezing at either a very fast or a very slow rate, followed by thawing, but not by freezing at an intermediate rate. No comparable lag has been observed in fish maintained in the frozen state at any temperature, regardless of freezing rate. In unfrozen cod, or in cod maintained at −7°C and below, a terminal inhibition to the "rapid" phase becomes evident at about 75% hydrolysis of the total phospholipids, and this is followed by extremely slow further breakdown. At −2.5 to −4.7°, however, the "rapid" phase appears to proceed to completion. Hydrolysis of phospholipids is promoted by the frozen state in two ways: a) abolition of any initial lag, b) increase of reaction rate. The reaction rate passes through a maximum at about −4°C and even at −7°C is faster than at 0°C.

INTRODUCTION

It was recently suggested (Dyer and Morton, 1956; Dyer *et al.*, 1956; Dyer and Fraser, 1959; King *et al.*, 1962) that hydrolysis of tissue lipids may be a factor involved in the denaturation of fish muscle protein during cold storage. While we consider that this hypothesis is not in harmony with certain experimental findings (Lovern, 1962a; Olley *et al.*, 1962), it does emphasize that post-mortem lipid hydrolysis is a current topic of potentially great significance to food technologists.

This paper, however, is not concerned with the significance of lipid hydrolysis. Rather, accepting as axiomatic that the aim in such a process as chilling or freezing is to retard change of any kind, attention is focused on the factors that may inhibit or promote hydrolysis of the lipids in the flesh of fish. It has been shown with the species most studied *(Gadus callarias)* that, even when bacteria are freely multiplying, lipid hydrolysis is not detectably influenced by bacterial enzymes (Olley and Lovern, 1960; Lovern, 1962a). Subsequent (unpublished) work in our laboratory has shown that this is not because of mechanical barriers to the bacteria, such as skin, since the rate of lipid hydrolysis is the same in eviscerated whole cod, skinned cod fillets, or even minced cod flesh all stored in ice. It is also known (Olley and Lovern, 1960) that non-enzymic chemical hydrolysis of the lipids does not occur in frozen cod. Thus, for this species it seems safe to postulate that the subject now under discussion relates solely to the enzymes of the flesh, and, in the absence of any evidence to the contrary, this is assumed to apply to all the species considered.

In an attempt to give an up-to-the-minute account of the present status of our work, which is still in progress, some data inevitably relate to experiments in which the conditions selected were not altogether the ones that later findings indicate as probably more informative. Such cases are indicated as they arise.

GENERAL CONSIDERATIONS

Two major groups of lipids will be distinguished for present purposes: triglycerides and phospholipids. The triglycerides

constitute an energy reserve that, in the flesh, fluctuates widely in amount from one species of fish to another, and seasonally within a species. The phospholipids, being concerned with cell structure and function, vary relatively little in concentration in the flesh, either seasonally or with species.

The enzymes that hydrolyze these two classes of lipid are lipase and a group of phospholipases. No evidence has been found for the presence in fish muscle of those phospholipases (C and D) that remove the nitrogenous base or the phosphate-base unit (Lovern, 1962a). The other two phospholipases (A and B) resemble lipase in that they hydrolyze the acyl esters of the glycerol portion of the phospholipid, liberating free fatty acids.

It should not be assumed that post-mortem autolysis bears any resemblance to events in living tissue. There, depot fat mobilization, for instance, is under nervous and hormonal control, and reaction products are removed by the blood stream. An example of lack of correlation between known events in the living fish and post-mortem autolysis is furnished by the herring *(Clupea harengus)*. This is a species that at certain seasons is depositing fat in the flesh at a high rate, but at other seasons is rapidly mobilizing this flesh fat. Yet in neither case is there any evidence of post-mortem lipase activity in this tissue (Olley and Watson, 1962).

TOTAL INHIBITION OF LIPID HYDROLYSIS

There are some species of fish in which no progressive post-mortem hydrolysis of one or other class of tissue lipid has been detected. This may be because the conditions under which the fish have been held are such as to inhibit lipolytic or phospholipolytic action in these particular tissues, and there are many variants of storage conditions that have not been tested. However, the conditions we have used have been such as would promote or permit lipid hydrolysis in other species of fish.

Three species of *Elasmobranch* fish, i.e., cartilaginous fish of the shark and ray group, have been studied. In complete contrast to thirteen species of *Teleostean* fish, i.e., bony fish, also studied by us, none of the Elasmobranchs showed any evidence of post-mortem hydrolysis of the flesh phospholipids. Chemically, the flesh phopholipids of a shark closely resemble those of a cod; they contain a similar mixture of various phosphatidyl esters. Yet, after, for instance, storage at −14°C for 16 weeks, no detectable hydrolysis of these phospholipids had occurred in either of two shark species or in a skate species, whereas in cod and most other Teleosts some 30–60% of the phospholipids had been hydrolyzed (Olley *et al.*, 1962) The hydrolysis rate was fairly similar in 11 species, but appreciably lower in the other two.

Virtually total inhibition of lipolysis of the flesh triglycerides has been observed in some species of fish, and there is no parallel in this respect with the findings on phospholipid breakdown. Naturally, lipase activity can be manifested only in species with triglyceride-containing flesh, whereas the flesh of all species contains phospholipids. The method we use to distinguish phospholipid hydrolysis from triglyceride hydrolysis, and to measure their relative importance when both occur simultaneously, depends on our finding that phospholipid breakdown in fish flesh involves virtually simultaneous liberation of both fatty acids from any phospholipid molecule attacked. Lysophosphatides do not accumulate to any detectable extent. In species lacking triglycerides in the flesh, e.g., the cod, a straight regression line can be plotted for free fatty acid production against the phosphorus content of the total lipids. The slope of this line is the same for all species in which phospholipids alone are being hydrolyzed.

If triglycerides (or other phosphorus-free lipids such as the alkoxydiglycerides of dogfish flesh) are hydrolyzed, free fatty acid will be produced without corresponding loss of lipid phosphorus. Accompanying phospholipid breakdown, this will affect the slope of the regression line referred to above. This is a convenient and experimentally simple way of detecting and estimating the separate contributions of triglycerides and of phospholipids to free fatty acid production. It does, however, depend on the universality of our finding that lysophospha-

tides never accumulate in significant proportions and that phospholipase D activity, leading to loss of lipid phosphorus without liberation of free fatty acids, is never manifested in fish flesh. So far we have not found any exception to these provisos.

Using this approach we have failed to detect any hydrolysis of the triglycerides of herring flesh, even in fish that in life must have been seasonally mobilizing this fat (Olley and Watson, 1962). The word "flesh" must be emphasized. We have found a slow but definite post-mortem lipolysis of triglycerides in the skin and immediately-underlying adipose tissue. The herring and the dogfish *(Squalus acanthias)* afford examples of the two extremes of total inhibition of hydrolysis of one class of lipid only. Both possess a flesh rich in phosphorus-free lipids—triglycerides in the herring and a mixture of triglycerides and alkoxydiglycerides in the dogfish. In the herring, autolysis affects only the phospholipids; in the dogfish, only the phosphorus-free lipids.

INITIAL INHIBITION OF LIPID HYDROLYSIS

With some species of fish, cod and herring being examples, inhibition of phospholipid hydrolysis is quite marked for some considerable period after death. No comparable observations have been made on triglyceride hydrolysis with a species in which this lipid is hydrolyzed, e.g., dogfish. As would be expected, the length of this "lag" period before the onset of rapid hydrolysis depends on temperature. With cod it is about 10 days at 0°C whereas at 20°C it must be less than 24 hr and, in fact, was not detected in our experiments at that temperature. Data on herring are sparse, but at 0°C the lag seems to be comparable with that for cod (Olley and Watson, 1962). We have not yet studied the effect of storage temperatures intermediate between 0 and 20°C. Tomlinson *et al.* (1960) were unable to detect any phospholipid hydrolysis (loss of lipid phosphorus) in the flesh of lingcod *(Ophiodon elongatus)* held at 0°C for up to three weeks. Hydrolysis of some kind of lipid (production of free fatty acids) does occur in the flesh of frozen lingcod at −12°C (Wood and Haqq, 1962), and the total lipid content (0.85%) implies that it is most probable that phospholipids are involved. Thus it is possible that at 0°C the lag for this species extends to at least three weeks, but that inhibition would not be permanent.

This lag at 0°C for cod and herring does not represent a period of total inhibition but rather a period when phospholipid breakdown is occurring very slowly. There is no definite break in the hydrolysis rate curve; rather does the rate progressively increase, more-or-less exponentially.

In contrast to the total inhibition referred to earlier, this initial inhibition cannot be due to any lack of a stock of enzyme in the tissue. One possible reason would be that in the intact cell the main stock of enzyme is kept apart from the bulk of the phospholipids of the cell, and only after cellular disorganization, e.g., as a result of autolytic proteolysis, could enzyme and substrate really come together. An alternative possibility might be that conditions in the intact cell are such that phospholipase activity is hindered, e.g., by an unfavorable local pH or ion concentration. Once again, non-lipid autolytic effects, or changes in membrane permeability, might eventually lead to favorable conditions for attack on the phospholipids.

We have investigated the effect of cellular damage on the lag period. Simple mincing of the tissue has no effect. The effect of freezing, followed by prompt thawing, has been found to depend on the rate of freezing. In work involving different individual fish it is essential to use the "paired fillet" technique unless the mean values from large numbers of fish are to be used. Otherwise, biological variation obscures the significance of anything other than major effects. One fillet from each fish is subjected to the special treatment under study, and the others form a control series.

The effects of freezing at three different rates are shown in Fig. 1 (a,b,c). In the first example individual fillets, loosely wrapped in polyethylene bags, were placed in cold storage at −14°C. The "thermal arrest" (about −1 to −5°C), measured by spear thermometer, was 6 hr. In the second example, fillets were frozen in an air-blast

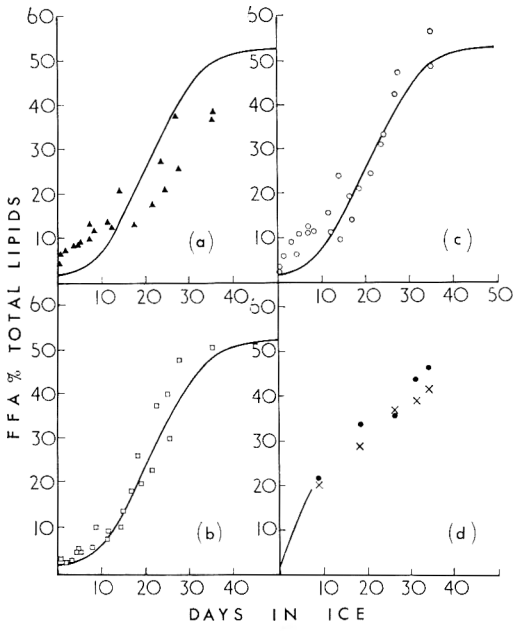

Fig. 1. Development of free fatty acids in the flesh of cod frozen at various rates, thawed, and stored in ice: (a) very slowly frozen, (b) frozen at an intermediate rate, (c) rapidly frozen, (d) frozen at intermediate rate and stored 7 days at −7°C. In (d) one set of paired fillets was thawed and stored in ice (× . . . ×), and the other set was left at −7°C (● . . . ●). Lines in (a, b, c) represent the statistical curve for all experiments with unfrozen fish in ice. Line in (d) indicates a common hydrolysis rate up to 7 days. The total lipid content of cod flesh is 0.67% (S.D.±0.09) (Olley *et al.*, 1962).

freezer running at −29°C, the thermal arrest being 22 minutes. The fastest freezing was achieved by surrounding individual fillets with finely-crushed solid carbon dioxide, giving a thermal arrest of about seven minutes. The more rapidly frozen fillets were held for two days in cold storage at −30°C before thawing, to make them more comparable to the more slowly frozen ones, which had remained overnight at −14°C after freezing. All fillets were then thawed overnight in a room maintained at 4°C, packed in polyethylene bags, and buried in crushed ice. Control fillets, in similar bags, were buried directly in crushed ice.

The experimental points shown in Fig. 1-a,b,c relate only to the frozen and thawed fillets. The lines represent a statistically plotted curve for cod stored in ice without any initial freezing, based on all the many experiments we have run with iced cod. The values for the paired fillets, which had already been in ice for two days before their frozen and thawed counterparts were placed in ice, fitted well with this statistical curve, but because of the time displacement were not so well adapted as the curve itself for comparison with the treated fillets. Values for individual paired unfrozen fillets did, however, confirm that the following picture is a consistent one.

In all cases, freezing resulted in a slight production of free fatty acids, a phenomenon we have observed before. The effect was particularly marked in the most slowly frozen fish. In these particular fish the lag appears to have been eliminated, but a curve drawn through the experimental points would cross that for untreated fish in ice soon after the end of the lag in the latter curve. Thereafter, hydrolysis is slower in the fish that have been frozen and thawed. This latter feature, consistently observed in other experiments, suggests damage to the enzyme by slow freezing even though favorable conditions have been promoted for initial hydrolysis

The intermediate rate of freezing has produced no significant effect other than a slightly higher initial level of free fatty acids. The lag remains and the curves do not cross, so that there is no evidence of enzyme damage. Very rapid freezing has abolished the lag, but does not appear to have damaged the enzyme.

This effect of freezing rate on the initial lag in lipid hydrolysis should be kept in mind in considering the course of hydrolysis in frozen fish. In our experiments at −7°C and below, the fish, or whole fillets, were air-blast frozen at a rate that should not have abolished the lag had they been promptly thawed again. Kept in the frozen state they showed no evidence of an initial lag. In experiments run at −2.5, −3.3, −4.0, and −4.7°C, it was desired to attain these temperatures rapidly since we were then primarily interested in the effect of temperature on hydrolysis rate in a region where this effect is very great (see below). For each run, two fish were filleted and the fillets sliced (about 6 mm thick) in a bacon slicer. From these slices, rectangular pieces of about 120 g were cut and closely fitted

into plastic bags that were immersed in appropriate freezing mixtures of ethanol-water, to which solid carbon dioxide was added to give a "slush" of ice and liquid. After freezing, the wrapped slices were transferred to 2-gallon Dewar flasks, fitted with heating elements, and placed in a cold chamber at −9°C. Thermostatic control was by contact thermometers in the flasks. It was virtually impossible to achieve accuracy of control to within 0.2°C, and in this temperature region such a variation could cause considerable scatter between the results of different runs. Freezing rate, not measured, must have varied over the range −2.5 to −4.7°C, which is within the thermal arrest zone. However, we did conduct two runs (at −4.0° and −4.7°C, respectively) with air-blast-frozen material (slices cut from frozen fillets), and in the series at −4.7° the paired fillet technique was used, i.e., one fillet was first air-blast frozen, the other sliced directly and immersion frozen. There was no apparent difference in subsequent hydrolysis between air-blast-frozen and immersion-frozen material. An earlier report of such a difference (Lovern, 1962b) is probably attributable to biological variability since different series of fish were involved.

Even with slices cut from one fish there will be biological variation, and with two fish (to give adequate material for a long run) this variability may be expected to increase. The results in Fig. 2 relate to two or three separate series at each temperature. Despite considerable scatter it is clear that at no temperature was there any initial lag comparable to that occurring with unfrozen fish at 0°C. Statistically, there is some suggestion of an initially slower hydrolysis rate in some cases, but it is far faster than during the lag phase at 0°C, and the curves do not depart appreciably from those for a first-order reaction (see below).

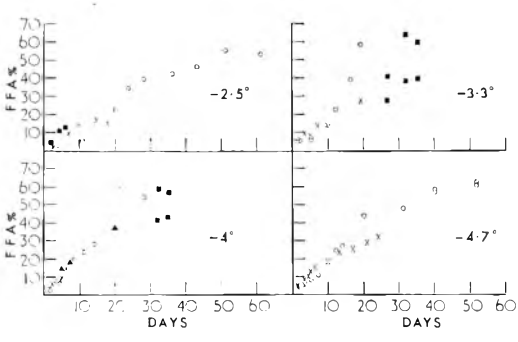

Fig. 2. Production of free fatty acids in cod flesh maintained at temperatures a little below the freezing point. Results of different experimental series at any one temperature are indicated by different symbols, but use of the same symbols for results at different temperatures does not imply direct comparison. The total lipid content of cod flesh is 0.67% (S.D.±0.09) (Olley *et al.*, 1962).

Despite the fact that the freezing rate at −2.5 and −3.3° was possibly fast enough to abolish the lag, it is known from comparable air-blast-frozen material that freezing rate was not the operative factor at −4.0 and −4.7°C. It appears that whatever may be the cause of the initial lag at 0°C, the conditions in frozen cod at any temperature are such that no lag of comparable significance occurs. An earlier report (Lovern, 1962b) of a lag of at least five days in cod stored at −1.4°C should be regarded with suspicion since whole fish were simply placed in a chamber at that temperature, and if freezing (as opposed to supercooling) occurred it would be extremely slow.

The production of some free fatty acids during the freezing and thawing cycle is probably attributable to the very rapid hydrolysis that occurs at temperatures a little below the freezing point. Even the rapidly-frozen fish spend some time in this temperature region during thawing. This subject is discussed later. Abolition of the lag by either slow or very fast freezing, contrasted with the lack of any such effect by a particular intermediate rate of freezing, may be understandable in the light of Love's (1958) findings that cell structural damage may be least at intermediate rates of freezing.

TERMINAL INHIBITION OF LIPID HYDROLYSIS

Under a wide variety of storage conditions, viz., unfrozen in ice, frozen at −7°C and below, or dry salted, hydrolysis of the phospholipids in cod slows down markedly after about 75% of the original phospholipids have been hydrolyzed. Examination of the remaining phospholipids shows no selective removal of particular fatty acids,

nor any discrimination between the two major components, phosphatidyl choline and phosphatidyl ethanolamine. Qualitatively, e.g., as revealed by chromatography on silicic-acid-impregnated paper, the remaining phospholipids closely resemble the initial mixture.

Bligh (1961) showed that although the phosphatidyl esters of choline and ethanolamine are hydrolyzed at comparable rates, two minor components, the phosphatidyl esters of serine and inositol, are apparently unattacked over 37 weeks at −12°C. Our own analytical data on the lipids after various periods of storage are not adequate for direct estimation of these serine and inositol esters, but we have found an indirect way of estimating them that confirms Bligh's observations. Briefly, the phosphatidyl esters of ethanolamine, serine, and inositol, together with polyglycerolphosphatides, can be titrated to phenolphthalein, in contrast to phosphatidyl choline. Knowing that phosphatidyl ethanolamine forms a roughly constant proportion of the total phospholipids over the greater part of hydrolysis, and already having data on the total titratable acidity of the phospholipids at all stages of hydrolysis in frozen fish, it was simple to make a proportional deduction from the titratable-acidity curve, leaving the fraction attributable to phosphatidyl serine, phosphatidyl inositol, and polyglycerolphosphatides. This has been done in Fig. 3, which shows that the latter value does not fall. Considering the roughness of the method, our value of about 6% is in fair agreement with Bligh's chromatographic curve, from which we have determined planimetrically that phosphatidyl serine and inositol together account for about 8% of the total phospholipids.

Here, then, is one reason why phospholipid hydrolysis might ultimately slow down and perhaps even cease before completion. But at the level of 75% hydrolysis, readily hydrolyzable phosphatidyl choline and ethanolamine still make up nearly 70% of the total phospholipids. Increasing competition for the enzyme by the other phospholipids might be a factor, although Dawson (1959) showed that phosphatidyl inositol and polyglycerolphosphatides actually activate phospholipase B, enabling it to attack lecithin instead of only lysolecithin. After the lecithin has gone, phosphatidyl inositol, for example, is itself attacked.

Although under many conditions phospholipid hydrolysis becomes extremely slow after about 75% of it has gone, we have observed that this is not so at temperatures between about −7°C and the freezing point of the tissue. It is also in this region that the over-all hydrolysis rate is greatest (see below). Our data relate to temperatures of −2.5, −3.3, −4.0, and −4.7. Our actual experimental runs have not been extended beyond about 85% hydrolysis (60% free fatty acids in the total lipids), but statistical examination reveals that the exponential hydrolysis rate curve asymptotically approaches 100% hydrolysis. This is in complete contrast to similar statistical treatment of the data at 20, 0, and −7°C or below, where the asympote of the curve is at about 75% hydrolysis. It is of interest to note that our statistician gave us these terminal

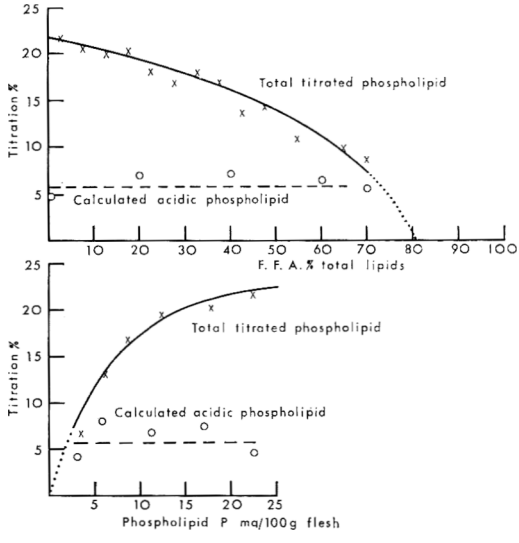

Fig. 3. Changes in phospholipids titratable to phenolphthalein during autolysis of frozen cod flesh. Results relate to all temperatures, and the crosses are means of all results aggregated to the nearest 5 units % of FFA and P. Total titrated phospholipids include phosphatidyl esters of ethanolamine, serine, and inositol, and also polyglycerolphosphatides. Titration units represent percentage of total lipids, calculated on a mean molecular weight of 800 and one titratable group in the molecule. For details of calculated acidic lipids, which are the total titratable lipids minus phosphatidyl ethanolamine, see text.

values, based on free fatty acid data, without knowing what the final level of fatty acids could be in the mixed lipids (including unsaponifiable matter) when all phospholipid had gone, viz., about 80%.

A formula for reaction kinetics that has been found empirically to fit all the temperatures we have used is a modified first-order reaction equation. The modification involves a factor *(r)* to take account of any initial lag. When there is no lag, r equals one. The formula is:

$$Y = A - B \exp [-(Kt)^r]$$

in which Y is the content of free fatty acids at time t, A is the asymptotic final level of free fatty acids, B is the difference between A and the initial level of these acids, and K the reaction rate. At −4.7°C and below, r is found statistically to be 1.0, whereas at 0°C it is about 2.2. At −2.5, −3.3, and −4.0°C the data in Fig. 2 suggest values for r in the range of 1.0–1.3.

Thus, at −2.5 to −4.7° there is no evidence that the reaction rate slows down because of accumulation of any less-readily hydrolyzable phospholipids. In this respect it is relevant to note that even at other temperatures the resistant fraction of the phosphatidyl choline and ethanolamine is attacked slowly. Although the asymptote of the first-order stage appears to be at about 75% hydrolysis, this stage is followed by a very slow further hydrolysis. Thus, in fish kept four years at −14°C (at which temperature the "fast" reaction is over in about one year), hydrolysis had proceeded to the extent of 85% of the total phospholipids. At this stage, of course, not all the phosphatidyl choline and ethanolamine have been destroyed, and, in fact, the data in Fig. 3 show that essentially only these two phospholipids had been attacked up to that time. At −2.5 to −4.7° the other groups of phospholipid must also be hydrolyzed without any appreciable break in the rate curve.

The lipids in the cell are present in various subcellular structures. Thus, after fish flesh is homogenized in a Waring blender in 0.25*M* sucrose, followed by differential centrifugation, some 60% of the phospholipids are associated with a fraction composed mainly of myofibrils, the rest being associated with the mitochondrial, microsomal, and supernatant fractions (Young, 1962). Assuming that this fractionation technique has not resulted in a whole series of artifacts so far as lipid distribution is concerned, a matter on which adequate experimental reassurance is desirable, it does raise the possibility that some of the lipid is less accessible to the enzyme. Chemically the lipid mixtures of each of these centrifugation fractions are closely similar, so that differential rates of hydrolysis according to location in the cell would not affect the over-all proportion of one phospholipid to another—specifically, the proportion of phosphatidyl choline to phosphatidyl ethanolamine. It could, however, conceivably explain the markedly lower rate of hydrolysis under most conditions after the 75% stage.

PROMOTION OF LIPID HYDROLYSIS

It has been shown that no appreciable initial lag occurs in phospholipid hydrolysis in frozen cod, and indeed, so far as our data go, this also applies to the frozen flesh of other species in which such hydrolysis occurs post-mortem. This is one kind of promotion attributable to the frozen state, and it should not be confused with the effect of the freezing process. Despite the importance of freezing rate, cod frozen at the intermediate rate and then stored in the frozen state do not show any lag.

Apart from its effect on the initial lag, it can further be shown that the frozen state greatly facilitates phospholipid hydrolysis in fish flesh. In the first place, despite the lowering of temperature, hydrolysis is faster at −7°C than at 0°C (Lovern, 1962a). Hence, some even stronger factor is opposing the normal retarding effect of a fall in temperature. To demonstrate that hydrolysis is really faster at −7°C than at 0°C it is desirable to circumvent the influence of the usual lag at the latter temperature, and to use the paired-fillet technique. It is not valid to compare calculated rate constants for reactions involving different kinetics. Paired fillets were frozen (by the method giving the intermediate rate discussed above) and then stored at

−7°C for one week. Thereafter one set of fillets was thawed and placed in ice, the other left at −7°C. The results of periodic withdrawal and analysis are shown in Fig. 1-d, where, despite the unexplained values for the third pair of fillets, the general picture is obvious.

Associated with this evidence for a promoting influence of the frozen state is the finding that hydrolysis rate does not progressively diminish with falling temperature after passing the point at which freezing commences. There is, of course, no definite freezing "point" for such a material as fish flesh. Some of the water in it will, in the absence of supercooling behavior, turn to ice at a little below 0°C. As the temperature is lowered, more and more water will be frozen while the concentration of solutes in the remaining water increases. At still-lower temperatures, various eutectic solutions will freeze and the composition of the remaining liquid solution will alter qualitatively as well as quantitatively. This means that enzymes and their substrates may be brought into closer relationship as a result of concentration, and also that ionic and co-factor conditions are altered. Whatever the explanation, studies of hydrolysis rate of phospholipids in cod flesh at temperatures of −2.5 to −4.7°C show that the rate passes through a maximum at about −4.0°C. The rate constants, calculated from the formula previously described, are shown in Fig. 4. Since there is no great variation in any case from a first-order reaction, it is valid to compare these constants. The enormous effect of temperature on rate in this temperature range should be noted. Below about −10°C the effect of temperature on rate is consistent and surprisingly large by comparison with the usual magnitude for reactions in unfrozen systems. Thus, over the range of −10 to −29°C, a fall in temperature of less than 3° is needed to halve the hydrolysis rate (Lovern, 1962b). When falling temperature actually increases the rate of hydrolysis, i.e., before the maximum is reached, there must be a particularly powerful promoting factor opposing this marked opposite effect of the temperature reduction itself.

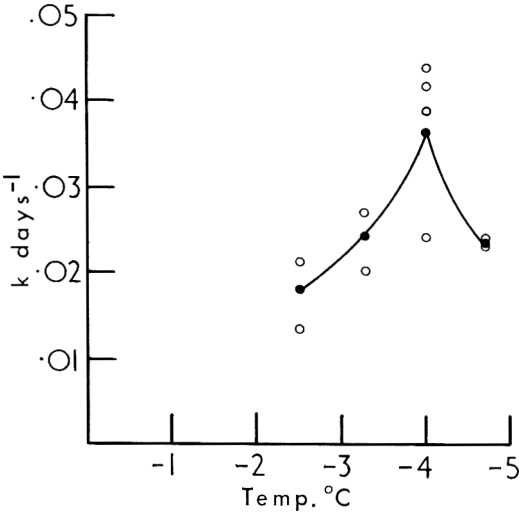

Fig. 4. Rate constants for hydrolysis of phospholipids in cod flesh at temperatures a little below the freezing point. Open circles represent calculations from individual experimental runs; closed circles represent mean values weighted statistically according to number of samples in the run and stage of hydrolysis, early stages being considered more significant.

It may be of interest to note that the temperature giving maximum phospholipid hydrolysis rate is not the same as that found by Love (1962) for development of toughness. But in both cases rate of change is maximum in the region a little below the freezing point, which has clear implications for the food technologist.

ACKNOWLEDGMENT

We are indebted to Mr. C. R. Baines for statistical evaluation of the work reported here, which was carried out as part of the program of the Department of Scientific and Industrial Research.

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Development and Application of an Apparatus for Continuous Measurement of Muscle Extensibility and Elasticity Before and During Rigor Mortis [a,b]

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SUMMARY

An apparatus was developed to study the extensibility and elasticity characteristics of muscle fibers before and during the onset of rigor mortis. A variable load (weight) was applied to a muscle specimen held in a chamber under controlled conditions maintained by a thermostatically regulated cartridge heater, cooling coil, and gas inlet. A solenoid cell, energized by a cyclic timer, was used to release and apply the load in a direction longitudinal to the vertically mounted specimen. A lever attached to the specimen-loading system transmitted the extensibility and elasticity of the specimen to the armature of a differential transformer. The output voltage from the secondary transformer winding was directly proportional to displacement of the armature. This AC output signal was rectified and transferred to a DC recording microammeter. The time course of rigor mortis was not influenced by loading and unloading interval or size of muscle strip, and was found to be temperature-dependent, being prolonged as temperature was decreased. The white fibers of the semitendinosus muscle were found to have a considerably longer delay phase than the red fibers. The relations between the time course of rigor mortis and the ultimate properties of the muscle were discussed.

INTRODUCTION

The onset of rigor mortis in muscle tissue has been extensively studied in rabbit (Bate-Smith, 1939; Bate-Smith and Bendall, 1949), horse (Lawrie, 1953), whale (Marsh, 1952), chicken (DeFremery and Pool, 1960), and beef (Marsh, 1954). Strips of parallel muscle fibers of specific dimensions were removed and subjected to extensibility and elasticity studies as described by Bate-Smith (1939) and Bate-Smith and Bendall (1949). The ends of the fibers were wrapped in adhesive tape and held in the jaws of clips normally used for making contact with terminals of car accumulators (Bate-Smith, 1939), and the loads were applied either manually or mechanically as described by Bate-Smith and Bendall (1949). DeFremery and Pool (1960) modified these extensibility measurements by sensing and recording electrically rather than mechanically.

The rigor mortis apparatus described in this paper was developed to study the time course of rigor mortis under various atmospheres, loads, loading intervals, dimensions of muscle strips, and changes in electrical resistance. It is planned to use this apparatus in further studies on muscle metabolism.

DESIGN OF APPARATUS

Fig. 1 is an over-all view of the "rigorometer" apparatus. The specimen chamber (Lucite) was

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[c] Dept. of Meat and Animal Science; ms. 360.

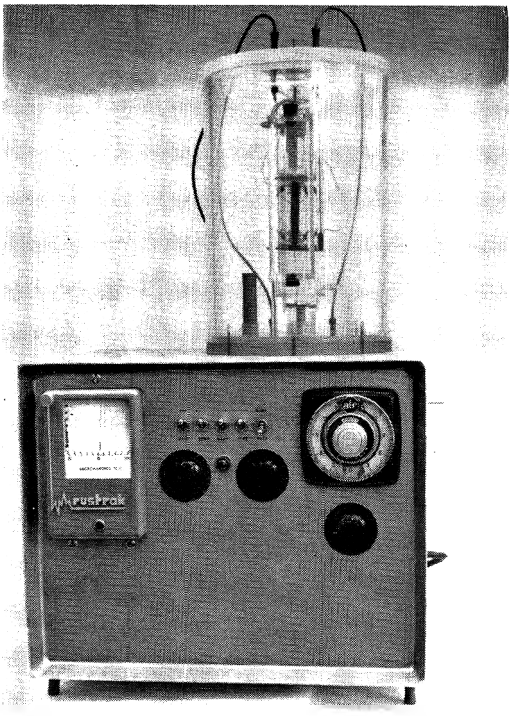

Fig. 1. Outside view of "rigorometer" apparatus.

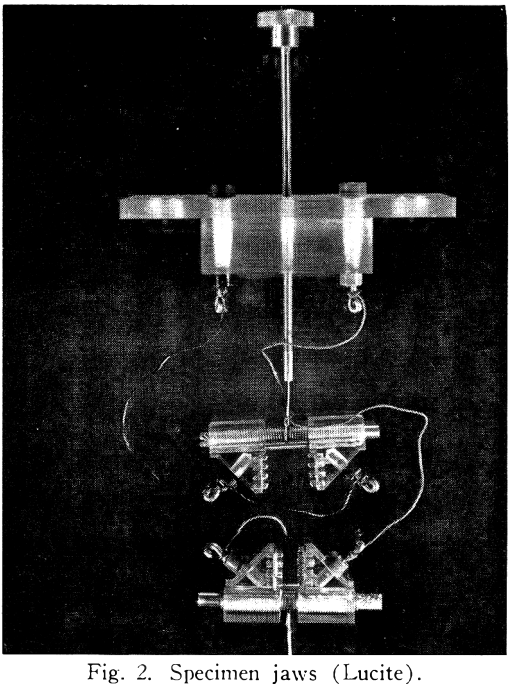

Fig. 2. Specimen jaws (Lucite).

sealed directly to the mechanism unit, and consequently required only limited laboratory space. Muscle strips were placed in Lucite jaws (Fig. 2) faced with stainless-steel prongs. These jaws were tightened to a fixed position, and this obviated taping the ends of the fibers to prevent slippage. The electrical resistance attachments were located on each jaw, making contact with the sample through the steel prongs. No additional time was required to make the proper attachment for this measurement. After the specimen was secured in the jaws, the entire assembly was inserted into the specimen chamber (Fig. 3). The specimen chamber was maintained under controlled conditions, independent of the laboratory environment, through use of a thermostatically regulated cartridge heater, cooling coil, and gas inlet. A small fan was placed at the bottom of an internal chamber that housed the heater and cooling coil. This provided uniform conditions throughout the sample chamber.

The wire attached to the bottom pair of jaws was connected to the armature of a solenoid cell through a small hole in the bottom of the chamber. The solenoid cell, energized by a cyclic timer (Fig. 4), was used to release and apply the load in a direction longitudinal to the vertically mounted specimen. A lever attached to the specimen-loading system transmitted the extensibility and elasticity of the specimen to the armature of a differential transformer. The output voltage from the secondary transformer winding was directly proportional to the displacement of the armature. This AC output signal was recti-

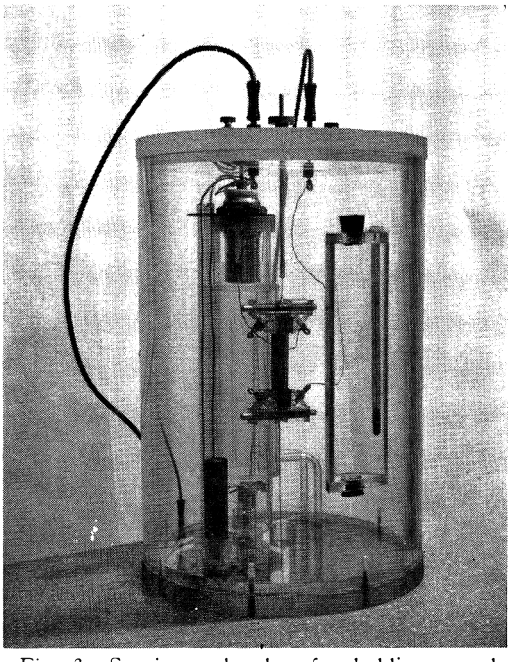

Fig. 3. Specimen chamber for holding sample under controlled conditions.

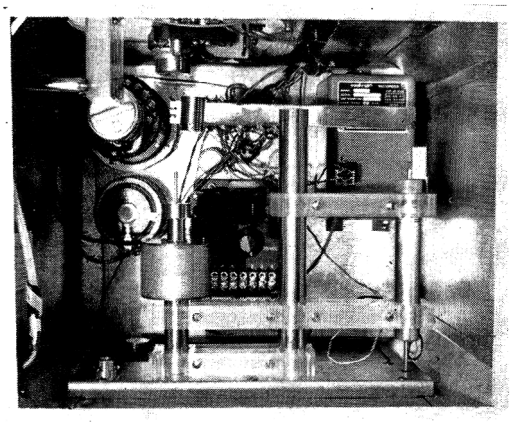

Fig. 4. "Rigorometer" mechanism unit.

fied and transferred to a DC recording microammeter. The prong-faced jaws of the specimen clamps also served as electrodes for making conductivity measurements. The initial resistance of the unloaded sample was balanced by a variable resistance. The change in electrical conductivity of the sample during the course of rigor mortis was indicated on the recording microammeter in units directly proportional to the actual values. Fig. 5 is the wiring diagram for this apparatus.

APPLICATION OF APPARATUS

Three phases of rigor mortis were determined on adjacent strips of parallel fibers (1 $cm^2 \times 8$ cm). A typical 2-min interval rigor mortis record is shown in Fig. 6. The "delay" phase (Bate-Smith and Bendall, 1949) represented virtually no

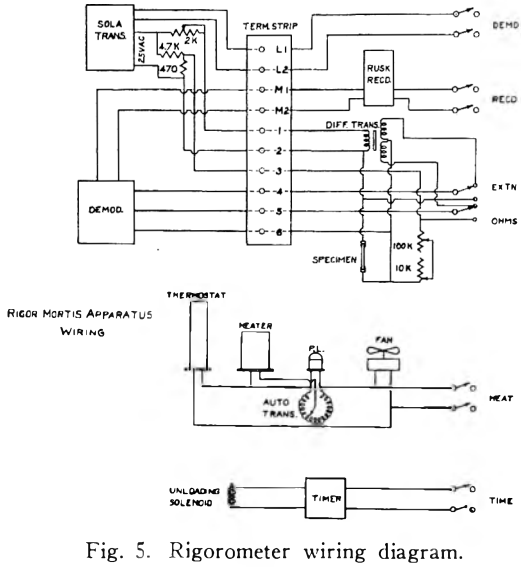

Fig. 5. Rigorometer wiring diagram.

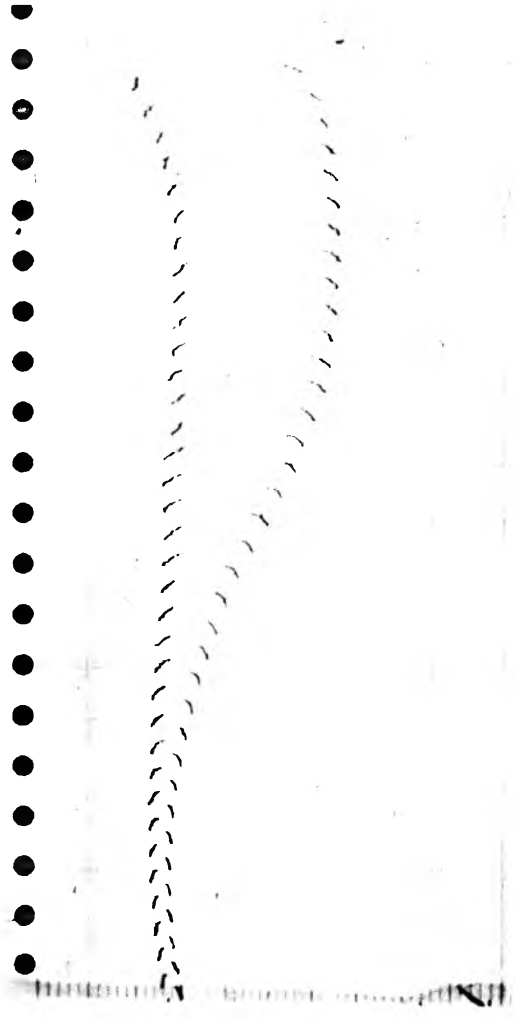

Fig. 6. Typical rigor record.

change in elasticity, whereas the onset phase represented a continuous reduction in elasticity. When all elasticity had disappeared, the muscle was considered in full rigor, which was termed the "completion" phase. The delay phase, onset phase, and completion of rigor mortis were easily detected. Fig. 7 is a typical record of the time course of changes in extensibility and elasticity compared to the time course of changes in electrical conductivity. This is in agreement with the report of Callow (1937), who found that electrical resistance determined at various port-mortem intervals followed much the same general course as the changes in elasticity. On the basis of the continuous record of electrical conductivity one could also de-

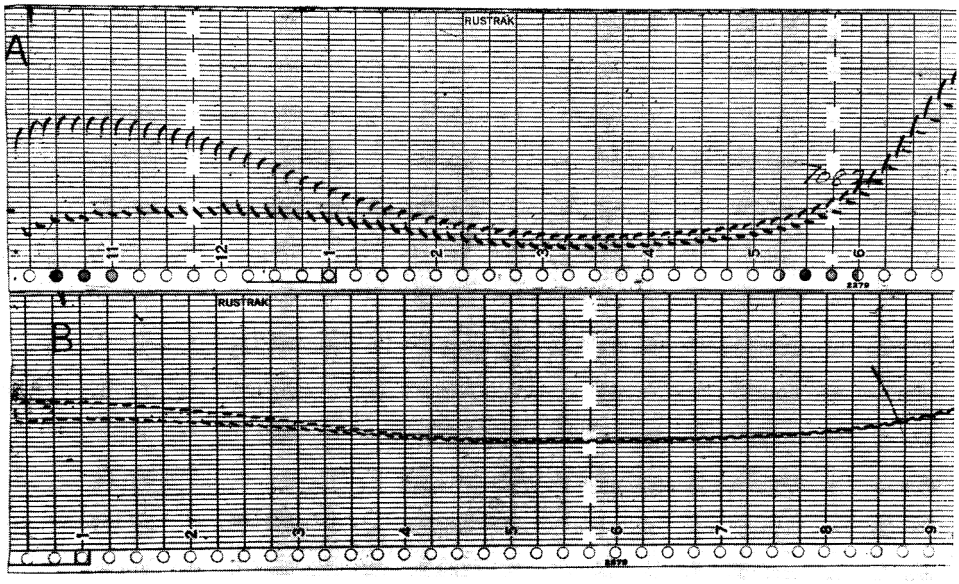

Fig. 7. Comparison of rigor record with changes in electrical conductivity. A) Rigor record; B) Electrical conductivity.

termine the delay, onset, and completion phases of rigor mortis. The determination of electrical resistance under different circumstances and its relation to changes in muscle properties will be discussed in detail elsewhere.

Muscle strips were dissected from the light portion (white fibers) of the semitendinosus muscle immediately after stunning (captive-bolt pistol) and exsanguination of the pig. Two "rigorometer" apparatuses were used to test the application of this equipment and the effect of various conditions on the time course of rigor mortis. Adjacent strips from the same muscle were used in each "rigorometer." One apparatus was maintained as a control at 37°C, 100% relative humidity, nitrogen atmosphere, and 50-g weights applied at 2-min intervals. One of the conditions of time interval, temperature, size of strip, atmosphere, or weight applied was varied in the second apparatus.

Loading interval. Variation in the time interval of load application and release (Fig. 8) of 1, 2, and 6 min as compared to 8 min, was not found to influence the time course of rigor mortis. Although the equipment described in this paper can load and unload the sample at intervals from a few seconds to 15 min, use of a 2-min interval made possible the specific determination of the rapid onset of rigor mortis in many of the muscles. Based on experience to date with approximately 100 pigs, the 2-min interval appears to provide a more definitive differentiation between the various phases than the 7–8-min interval

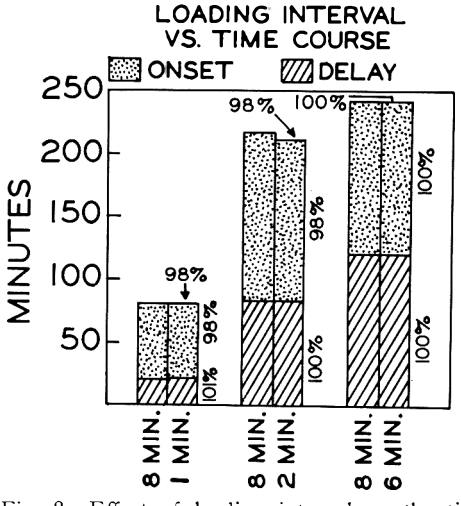

Fig. 8. Effect of loading interval on the time course of rigor mortis. "Delay" phase: no change in elasticity. "Onset" phase: continuous reduction in elasticity. Each phase is shown as a percentage of the time for its respective phase in the control apparatus.

used by other workers. Since the onset of rigor mortis was found to be extremely variable between pigs, ranging from 2 min to 8 hr, and since there were no significant differences between the phases of rigor mortis when the weights were loaded and unloaded at different time intervals, the 2-min interval was selected for the remainder of these studies.

Temperature variation. For study of the effects of temperature, the experimental rigorometer was used at temperatures of 43, 25, 21, and 10°C (Fig. 9). Results from

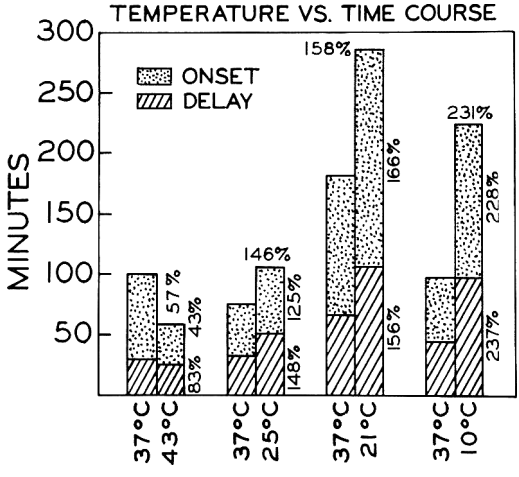

Fig. 9. Effect of chamber temperature on the time course of rigor mortis. Each phase is shown as a percentage of the time for its respective phase in the control apparatus.

the temperature study support several previous reports on the acceleration of glycolysis with increasing temperature (Bate-Smith, 1939; Bate-Smith and Bendall, 1949; Bendall, 1951; DeFremery and Pool, 1960; Lawrie, 1953; Marsh, 1954). DeFremery and Pool (1960) found that the time required for 50% reduction in initial ATP concentration was 50% less at 43 than at 40°C. It was found in the present study that the duration of the onset phase and total time for completion of rigor mortis at 43°C were markedly reduced (to 45% and 57%) when compared to the rates at 37°C. This parallels the findings of Bate-Smith and Bendall (1949) with rabbit muscle. The total time for rigor completion was 50% longer at 25°C than at 37°C, 50–60% longer at 21°C, and approximately 130% longer at 10°C. Bate-Smith and Bendall (1949) found that lowering the temperature from 37 to 17°C decreased the delay phase about 2.5 times and had an even larger effect on the "rapid" phase. Since most semitendinosus porcine muscles approached the onset phase within 60–90 min, when the normal internal temperature of the ham would have been 35–40°C, it seemed that one could more nearly simulate the normal condition by conducting the extensibility and elasticity measurements at 37°C.

Weight of load, strip size, and atmosphere. Most of the previous reports indicate the use of a 50-g weight at 8-min time intervals. Fig. 10 shows results of this study on the effect of weight on the duration of the delay and onset phase. Use of a 125-g weight prolonged the delay phase and accelerated the rapid phase, while the use of a 25-g weight was found to reduce the duration of both the "delay" and "onset" phases by approximately 10% (Fig. 10). As shown in Fig. 11, quadrupling the cross-sectional area of the strip had no significant effect on the time course of rigor mortis. Conversely, the rigor mortis process was approximately 25% longer in an oxygen

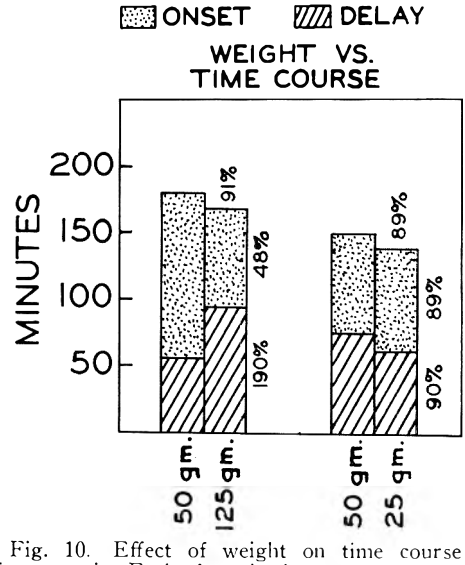

Fig. 10. Effect of weight on time course of rigor mortis. Each phase is shown as a percentage of the time for its respective phase in the control apparatus.

atmosphere than under nitrogen (Fig. 11). This is in support of the work of Bate-Smith and Bendall (1949), who showed a pronounced delay in the onset of rigor when conducted under oxygen.

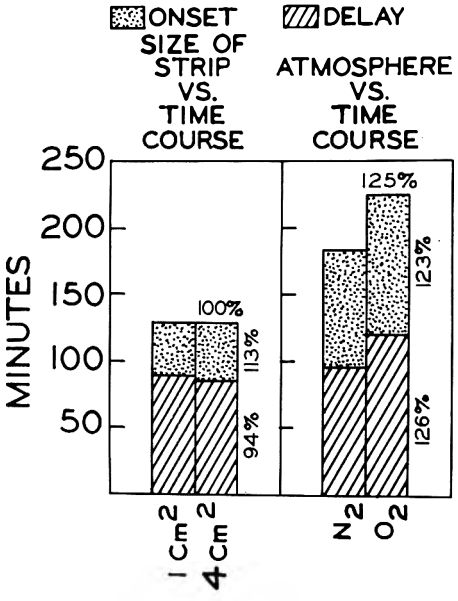

Fig. 11. Effect of size of strip and atmosphere on time course of rigor mortis. Each phase is shown as a percentage of the time for its respective phase in the control apparatus.

Rigor mortis in various muscles. All measurements were made under the standard conditions previously described. The time course of rigor mortis was determined in two muscles from each of ten pigs. Muscle strips of the light portion (white fibers) of the semitendinosus, dark portion (red fibers) of the semitendinosus and longissimus dorsi were used in these studies.

A comparison of the time course of rigor between dark and white fibers of the semitendinosus (Fig. 12) support the work of Lawrie (1952). The delay phase of the red fibers was only one-fourth as long as the same phase in the white fibers. The red fibers had a prolonged onset phase but the total time required for completion was reduced by about 40% when compared to the white fibers. The longissimus dorsi had a prolonged delay phase when compared with either the light or dark sections of the semitendinosus. This would seem to reflect intrinsic differences in the metabolism of the muscle and character of the tissue rather than a pigment difference.

Rigor record vs. muscle quality. Forty-eight pigs of known breeding and nutrition were utilized in this study. The muscle strips were removed immediately after stunning and bleeding and were placed in the rigor mortis apparatus within 8 min of death. The pH values, subjective color scores, and objective color measurements were determined at various post-mortem intervals according to the procedures described by Sayre *et al.* (1961).

The relations of four types of rigor mortis patterns to muscle properties are shown in Fig. 13. Both the rapid (10-min delay phase) acid rigor (A) and moderately slow (60-min delay phase) acid rigor (B) resulted in pale, soft, watery tissue. Normal tissue (firm and dry) was associated with the short delay, moderately long onset phase (C) when the pH of the muscle at 45 min remained high. A long "delay" phase and an extremely long onset phase in (D) resulted in dark firm muscle that had virtually no shortening after completion of the rigor mortis process.

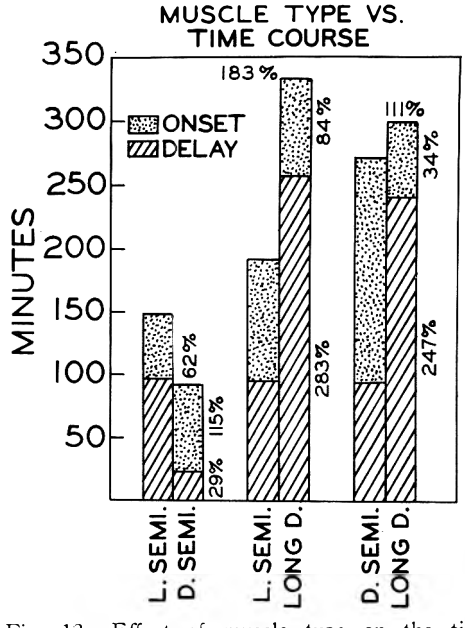

Fig. 12. Effect of muscle type on the time course of rigor mortis. L. Semi = light section semitendinosus; D. Semi = dark section of semitendinosus; Long D. = longissimus dorsi.

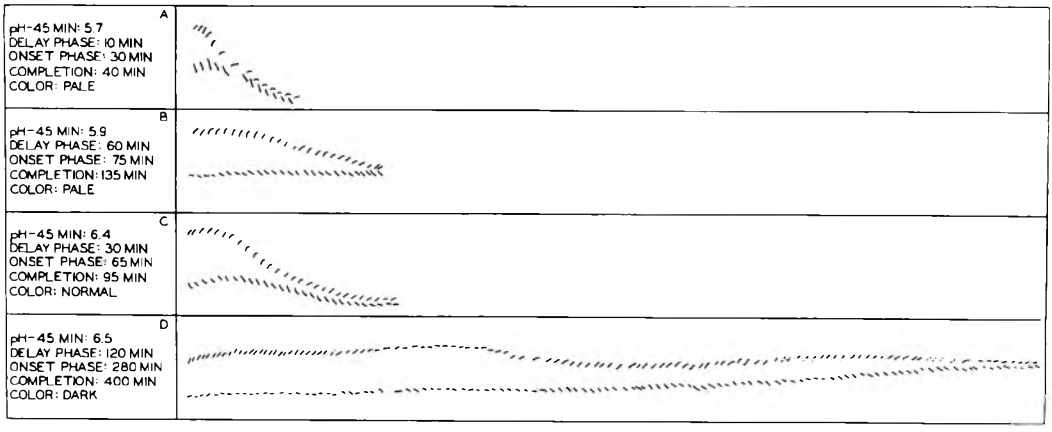

Fig. 13. Effect of time course of rigor mortis on pork muscle appearance.

Other examples of rapid "acid" or "alkaline" (Bate-Smith and Bendall, 1949) rigor mortis showed severe shortening (40%) and subsequent lengthening to 100–200% of their original length. These muscles appeared to be dark, moderately light, or pale, depending not only upon the pH of the muscle at the onset of rigor but also upon the duration of the onset phase. It is therefore postulated that many of the variations in pork muscle properties can be associated with the time course of rigor mortis.

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Factors Affecting Heat Inactivation and Partial Reactivation of Peroxidase Purified by Ion-Exchange Chromatography [a,b]

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SUMMARY

Diethylaminoethyl-cellulose (DEAE) was used in an ion-exchange chromatographic fractionation of commercially prepared horse-radish peroxidase. One major fraction was separated from other minor fractions and used for heat inactivation and reactivation work. Heat inactivation was carried out at 250–300°F, with most of the inactivation work at 280°F. Peroxidase activity was assayed by measuring the rate of utilization of hydrogen peroxide in the formation of tetraguaiacol from guaiacol. The concentration of tetraguaiacol was measured with a recording spectrophotometer. The ionic strength of the buffer solution in which the peroxidase was heated had an effect on its inactivation. Within limits, the higher the ionic strength the greater the heat inactivation of the enzyme. Activity was maximum at pH 7. Purified peroxidase heated 30 sec at 280°F and stored 24 hr at 72°F, with the heat treatment and storage time repeated three times, decreased in activity as a first-order reaction. The storage temperature of heat-inactivated peroxidase affected the rate and degree of reactivation.

INTRODUCTION

Canned foods may be held at room temperature with essentially no adverse effects from enzyme activity because the customary heat treatment used to sterilize the food is sufficient to inactivate enzymes in the tissues. However, enzyme activity in high-temperature short-time (HTST) canned vegetables has been demonstrated. In much of the work involving enzyme activity and HTST processing it has been noted that a negative test for peroxidase immediately after processing was soon (sometimes within 24 hours) followed by a positive test. This phenomenon has been termed a regeneration or reactivation of the enzyme peroxidase.

Guyer and Holmquist (1954) reported that HTST-canned peas developed within eight months, an off-flavor that they attributed to enzyme activity even though the product had been processed sufficiently to sterilize it. Vettor *et al.* (1959) demonstrated peroxidase regeneration in whole-kernel sweet corn processed at 240–300°F. Esselen and Anderson (1956) presented data on the thermal destruction of peroxidase in seventeen different vegetables at 215–300°F and showed that the degree of heat required to prevent regeneration of enzyme activity during storage was 2-4 times as great as that required to destroy the enzyme on a basis of tests made immediately after heating. Farkas *et al.* (1956), working with peroxidase from crushed fresh peas, found that regeneration of this enzyme becomes a factor only when the required heat-inactivation time is greater than that provided by normal processing, e.g., above 255°F. Schwimmer (1944) showed that regeneration of peroxidase in turnip and cabbage juice is a function of time and temperature. In a comprehensive review on the thermal inactivation of food enzymes, McConnell (1956) stated that peroxidase appears to be the most heat-resistant enzyme found in vegetables. Adams and Yawger

[a] Approved for publication as Technical Paper 1582, by Director of Oregon Agricultural Experiment Station.

[b] Presented at 22nd annual meeting of the Institute of Food Technologists, Miami Beach, Florida, June 11, 1962.

(1961) determined peroxidase activity and color over a 4-week storage period on peas processed at 230–280°F, and reported that the lethality of the process required to inactivate the enzymes was 10 times as great at 280° as at 250°F.

Since peroxidase can be obtained in a highly purified state from horse-radish roots, much work has been done on the kinetics of this enzyme system. Chance (1943, 1949a,b) determined the intermediate enzyme-substrate compounds and studied the kinetics of the reactions of these compounds with hydrogen donors, using a rapid-flow spectrophotometric apparatus. Maehly (1955a) studied the recombination of equimolar amounts of protohemin and apoprotein of horse-radish peroxidase in aqueous solution by spectrophotometric techniques.

The present work was undertaken to further study the effects of heat in the range of HTST canning (250–300°F) on highly purified peroxidase. It is hoped this will lead to methods by which enzymes in foods may be irreversibly inactivated without overheating the food.

MATERIALS AND METHODS

Enzyme. The horse-radish peroxidase was a commercial preparation obtained from Worthington Biochemical Corporation, New Jersey. The principal fraction obtained from cellulose ion-exchange chromatography was used in this investigation. The enzyme, either in various solutions or in the dry crystalline form, was stored at 34°F.

Heat treatment of peroxidase. A 50-ml beaker was partially filled with the enzyme solution, and capillary tubes, open at both ends, were placed in the beaker in a near-vertical position. Capillary tubes were withdrawn singly, and the upper end (approximately 20 mm from the contained solution) was heat-sealed in a small gas flame. After all the tubes in the beaker were thus sealed at one end, they were inverted and slowly centrifuged to drive the solution to the sealed end, and the remaining open end was then sealed. Controls were run in which the activity of the enzyme in the beaker was compared with that in the sealed tubes before heat treatment. Sealing the capillary tubes in this manner produced no apparent effect on the activity of the enzyme. The capillary tubes were approximately 68 mm long, 0.8 to 1.1 mm ID, with a wall thickness approximately 0.25 mm. Groups of from 4–10 sealed capillary tubes were suspended at one end in partially extended metal springs mounted below wooden rods in such a manner that when the rods were placed across the open, hot glycerine bath (approximately 10-L capacity) the capillary tubes and metal springs were completely submerged in the heating medium. Different periods and temperatures (250–300°F) were used as heat treatments. The temperature of the glycerin bath (continuously agitated with a submerged propeller was controlled (±0.2°F) by a thermoregulator. Timing was done with a stop watch, and cooling was effected by plunging the tubes into cold tap water.

The enzyme solution was removed from the capillary tubes (usually four per assay) by breaking both ends and blowing the solution into a small vial, from which a syringe was filled and the proper amount delivered to the cuvette.

Peroxidase assay. The guaiacol test of Devlin was used as reported by Chance and Maehly (1955). The activity of peroxidase was measured as the rate of utilization of hydrogen peroxide (substrate) to form the colored product tetraguaiacol from guaiacol (hydrogen donor). The H_2O_2 was analytical reagent-grade 30%, diluted with distilled water prior to use. The N.F. guaiacol was redistilled at 205°C. The buffer solution, pH 7.0, consisted of a 10m*M* mixture of solutions of Na_2HPO_4 and KH_2PO_4. A Beckman DK-1 recording spectrophotometer was used, equipped with a constant-temperature cell holder that allowed for continuous circulation of water at 20°C. Assays were run at 470 mμ with silica cells having a 10-mm light path.

In all assays, the reaction cuvette contained 2.95 ml of buffer, 0.05 ml of 20m*M* guaiacol, 0.01 ml of 40m*M* H_2O_2, and 0.05 ml of the test peroxidase solution, giving a total of 3.06 ml in the cuvette. The buffer and guaiacol solutions were mixed 2.95:0.05 v/v in quantities of 200–300 ml and placed in the 20°C water bath before all assays. Three ml of this solution were then placed in the reaction cuvette immediately prior to placing the cuvette in the cell holder of the instrument. The peroxidase solution was added to the cuvette by a 50-μl syringe. The H_2O_2 solution was added to the cuvette after it had been placed in the cell holder of the instrument by delivering 0.01 ml from a 10-μl syringe to the tip of a small stirring rod, which was used to stir the contents of the cuvette vigorously for about 3 seconds. Then the recorder was turned on and the continuing change in absorbance was recorded as a straight line.

Used to determine e, the molar concentration of peroxidase, was the equation reported by Chance and Maehly (1955):

$$e = \frac{\Delta x}{a_0 \cdot k_4 \cdot \Delta t}$$

where $\Delta x = 7.5 \times 10^{-6} M$, a constant representing the H_2O_2 utilized in the formation of tetraguaiacol

$a_0 = 3.27 \times 10^{-4} M$, the concentration of guaiacol in the cuvette before the reaction starts

$k_4 = 2.2 \times 10^5$ at 20°C, the velocity constant for the reaction of the secondary complex with the hydrogen donor molecule

Δt = seconds required for the absorbance to increase 0.05 as measured from the spectrophotometer recording at 470 mμ.

Enzyme fractionation. DEAE-cellulose, type 20, 0.93 milliequiv/g was purchased from the Brown Company, Berlin, New Hampshire. Before use, the cellulose was washed by the procedure of Mandeles (1960). It was then suspended in water for several hours to allow trapped air bubbles to escape, and the remaining fines were decanted. The treated cellulose was then suspended in 0.1N NaOH, and portions were poured into a glass chromatographic column (2.6 cm inside diameter by 40 cm long). The settled column of cellulose was 26.5 cm long. At this time, the column was washed to neutrality with water, followed by a wash with 0.02M glycine until a blue band was washed out of the column.

In the fractionation (Fig. 1), 100 mg of crystalline peroxidase were added to 10 ml of 0.02M glycine, and the resulting solution was allowed to sink almost entirely into the column before the non-linear gradient elution was begun. The non-linear gradient elution schedule was the same as that of Mandeles (1960). The mixing flask at the head of the column contained 750 ml of 0.02M glycine, and the reservoir contained 750 ml of 0.02 mole each of KH_2PO_4, K_2HPO_4, and glycine per liter of solution. The flow rate was adjusted to 2 ml per minute. When the solution in the reservoir was exhausted, it was replaced by 750 ml of a solution containing 0.1 mole KH_2PO_4, 0.1 mole NaCl, and 0.02 mole glycine per liter of solution. When this solution was used up, the reservoir was filled with 750 ml of solution containing 0.1 mole KH_2PO_4, 0.1 mole NaCl, 0.03 mole HCl, and 0.02 mole glycine per liter of solution. Ten-ml fractions were taken from the column, and absorbance was determined at 280 mμ. When the absorbance at this wavelength showed an increase, the absorbance on the particular sample was also run at 403 mμ (the Soret band) as an indication of the porphyrin ring of peroxidase.

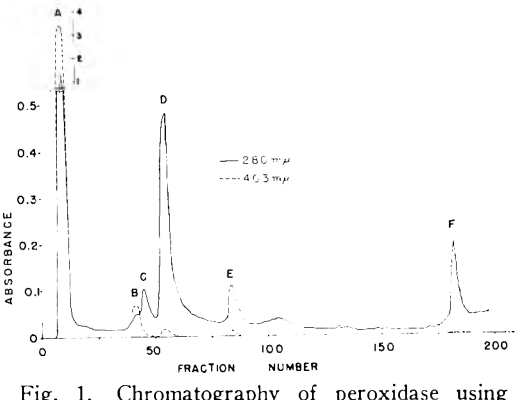

Fig. 1. Chromatography of peroxidase using DEAE-cellulose and non-linear gradient elution.

RESULTS AND DISCUSSION

Tube numbers 8, 9, and 10 of the peroxidase ion-exchange separation (fraction A of Fig. 1) had respective ratios of 1 to 2.43, 2.48, and 2.46 of absorbance at 280–403 mμ. In the range of 260–270 mμ the absorbance spectra of these fractions were essentially identical. These data indicate very good uniformity in those three fractions of the first and largest portion that came through the column. These three fractions were combined, labeled fraction A, and used in all subsequent experimental work except where noted.

Peroxidase activity was found in all the peaks shown in Fig. 1 where a measurable increase in absorbance was noted at 403 mμ. The molar concentration of peroxidase as determined by the guaiacol assay, which measures activity, was compared to the concentration as calculated by the molar absorptivity of 89.5×10^3 (Maehly, 1955b) and absorbance at 403 mμ. These results are shown in Table 1.

A solution of unfractionated peroxidase was made by weighing 0.0088 g into a 25-ml volumetric flask and filling with distilled water. Using the molar absorptivity of 89.5×10^3, a concentration of $5.69 \times 10^{-6} M$ was calculated for this solution, which gave an absorbance of 0.509 at 403 mμ. This solution was then diluted 1:10, sealed in capillary tubes with the previously described procedure, and heated at different tempera-

Table 1. The molar concentration of peroxidase of five peaks separated by DEAE-cellulose-column chromatography comparing assayed concentration with calculated concentration using absorbance at 403 mμ and the molar absorptivity of 89.5×10^3.

Tube no.	Peak	Absorbance at 403 mμ	Molar concentration of peroxidase	
			Assay by guaiacol method	Calculation by molar absorptivity
9	A	3.428	47.61×10^{-6}	38.31×10^{-6}
41	B	0.067	38.98×10^{-8}	74.97×10^{-8}
45	C	0.022	14.14×10^{-8}	24.60×10^{-8}
54	D	0.018	8.87×10^{-8}	20.13×10^{-8}
84	E	0.013	1.03×10^{-8}	14.50×10^{-8}

tures for varying periods. Fig. 2 shows the results in duplicate of the 60-sec heating. These data indicate that the concentration of peroxidase (measured by its activity) decreased in a first-order reaction rate when heated 60 sec at 250–300°F.

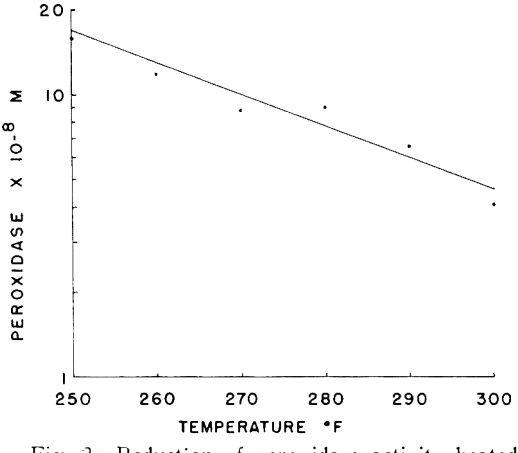

Fig. 2. Reduction of peroxidase activity heated 60 seconds at indicated temperatures with an initial concentration of 56.9×10^{-8} M.

To confirm the optimum activity at pH 7 as reported by Chance and Maehly (1955), 10m*M* buffer solutions were made up with enzyme concentrations ranging from approximately 45 to $90\times10^{-10}M$ in the assay cuvette. The activity of the enzyme was determined at the following pH values: 4.76, 5.60, 6.12, 6.69, 7.08, and 7.80. The results (Fig. 3) indicate that enzyme activity was optimum at pH 7 under these conditions.

Four phosphate buffer solutions of pH 7 were made having ionic strengths of 0.01, 0.03, 0.10, and 0.30. Fraction A peroxidase (Fig. 1) was diluted in these buffers in an approximate concentration of $45\times10^{-8}M$. The buffer used for assay purposes in the cuvette in the spectrophotometer had an ionic strength of 0.10. Each of the four different-ionic-strength enzyme solutions was heat-sealed in capillary tubes and heated at 280°F for 0, 5, 10, 30, and 60 seconds. Then they were immediately cooled and assayed for enzyme activity. The results (Fig. 4) indicate that at 0 heating time, the greatest activity was at the lowest ionic strength. The two lowest-ionic-strength solutions imparted a greater heat resistance

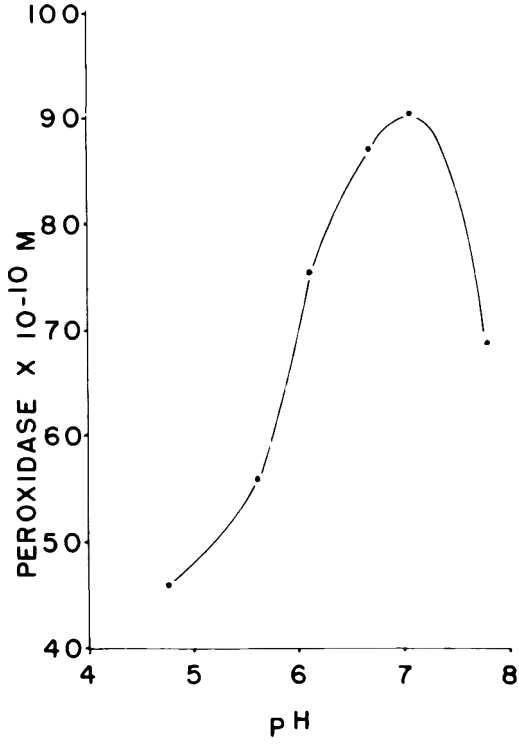

Fig. 3. Activity of peroxidase at different pH values.

to the enzyme solution than did the higher-ionic-strength solutions.

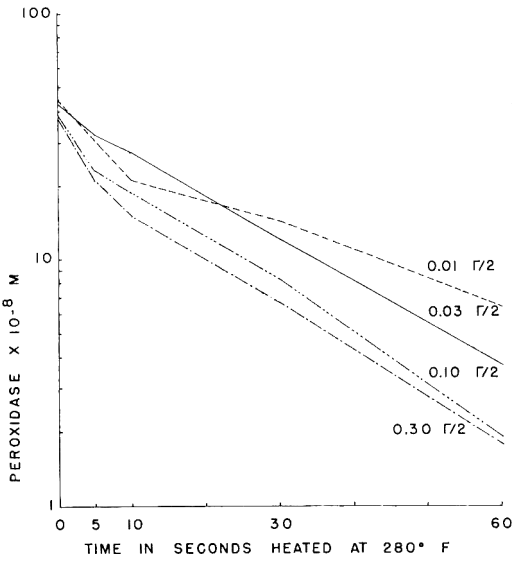

Fig. 4. Reduction of peroxidase activity heated at 280°F for different lengths of time in buffers of different ionic strengths.

To determine the effect of varying ionic strengths during assay of the enzyme in the spectrophotometer cuvette, the following experiment was carried out. Fraction A peroxidase was diluted in buffer of 0.01 ionic strength, and samples of this dilution were assayed in 4 ionic strengths of 0.01, 0.03, 0.10, and 0.30 as the buffer in the cuvette. The results (Fig. 5) indicate the peroxidase concentration in the cuvette. It is apparent from these results that the lower the ionic strength the greater the activity of the enzyme.

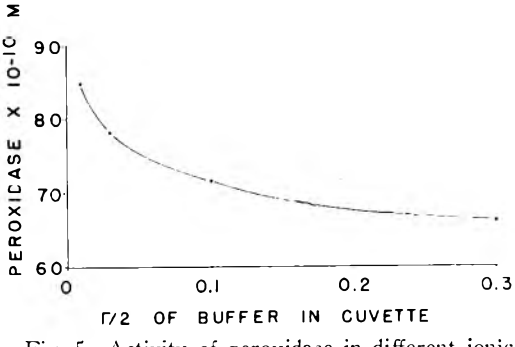

Fig. 5. Activity of peroxidase in different ionic strength buffers of the assay solutions.

The reactivation of heat-inactivated peroxidase stored at different temperatures and for varying lengths of time was also determined. Peroxidase fraction A was diluted 1:100 with 20mM phosphate buffer at pH 7.0, resulting in an assayed concentration of $48.3 \times 10^{-8}M$. This enzyme preparation was sealed in sterile capillary tubes with the standard procedure and divided into several lots. Aseptic procedure was not used, but methods were used to hold microbial contamination to a minimum. Separate lots were heated for 0, 5, 10, 30, and 60 seconds. Samples of all lots, after heating were cooled to 34, 72, and 100°F and placed in storage at those temperatures. The concentration of each lot was determined immediately, and again at the end of the following periods of storage: 1/3 day and 1, 5, and 9 days. This experiment was repeated but all tubes were cooled to 34°F after heating, held at this temperature for 2 hr, then stored at 72 and 100°F for the same periods as the samples cooled to their respective storage temperatures. There was apparently no effect of cooling to 34° before raising to the respective storage temperatures. The activity of the unheated enzyme declined somewhat during the first day of storage at all three temperatures, and then slowly regained its activity up to approximately its starting concentration of $48.3 \times 10^{-8}M$ except in the samples stored at 100°F. These had a steady decline during the 9-day period, from 48.3 to approximately $30 \times 10^{-8}M$, indicating that the enzyme preparation diluted under these conditions and stored at 34 and 72°F was relatively stable but that the activity steadily declined when the temperature was as high as 100°F.

Fig. 6 graphs the results of averaging the data and then calculating the percent reactivation. It can be noted that after a partial heat inactivation at 280°F and subsequent storage at three different temperatures the greatest reactivation occurred within the first 8 hr, regardless of the storage temperature. The reactivation continued through the first day for all samples. Samples stored at 72°F continued to reactivate through a 9-day storage period. Samples stored at 34°F continued a slight increase in reactivation after the first day, and samples stored

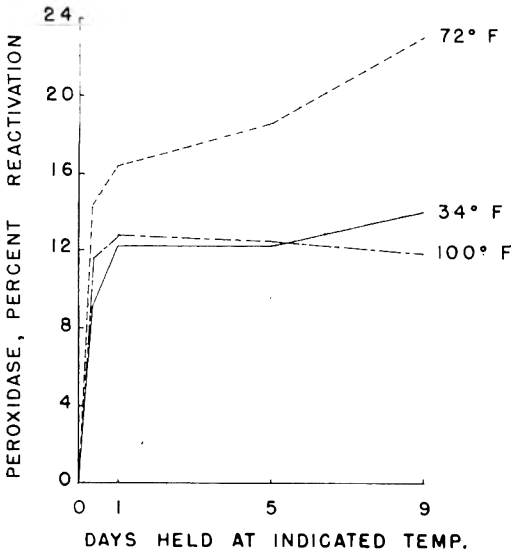

Fig. 6. Percent reactivation of heat-inactivated peroxidase held at different temperatures.

at 100°F showed a maximum reactivation at 1 day and then a slight gradual decline in activity to 9 days.

Fraction A of the peroxidase enzyme was diluted with buffer at pH 7.0 and 0.01 ionic strength. In all assays in this particular experiment, buffer of 0.01 ionic strength was also used in the assay reaction cuvette. The capillary tubes were filled with the enzyme solution and sealed. The unheated enzyme dilution was then assayed and found to be $86.8 \times 10^{-8}M$. Immediately afterward, all tubes were heated 30 seconds at 280°F, and a sample immediately assayed was found to be $37.3 \times 10^{-8}M$. All of the heated tubes were then stored 24 hr in a water bath at 72°F. Then a sample of the tubes of enzyme solution was found to be $43.1 \times 10^{-8}M$, showing an increase of 5.88×10^{-8} moles. Immediately after assay, all the remaining tubes were again heated 30 seconds at 280°F, assayed, and found to be $14.8 \times 10^{-8}M$, a loss of 28.3×10^{-8} moles, and put in the 72°F water bath for another 24 hr, during which the activity increased by 6.0×10^{-8} moles. The third heat treatment reduced the activity by 14.8×10^{-8} moles, but the third day of storage reactivated 3.96×10^{-8} moles. The fourth heat treatment reduced activity by 7.48×10^{-8} moles, but the fourth day of storage reactivated 2.20×10^{-8} moles. The ratio of moles of active enzyme lost by heating to the moles reactivated in 24-hr storage periods declined over a four-day period: 8.42, 4.71, 3.73, and 3.40. The quantity of enzyme reactivated became greater in proportion to the quantity inactivated by the heat treatment. The data are graphed in Fig. 7.

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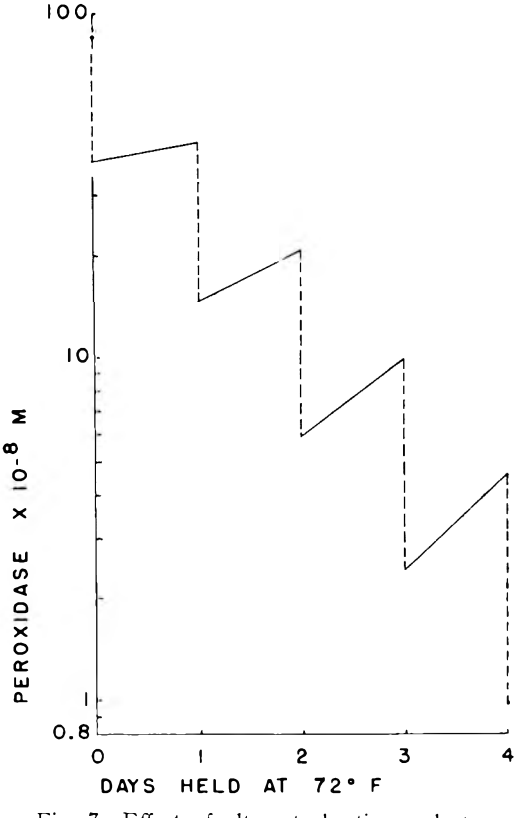

Fig. 7. Effect of alternate heating and storage on peroxidase activity.

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Darkening of Red Beets, Beta Vulgaris

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SUMMARY

Color changes in disrupted tissue of red beets were studied under various conditions with a Hunter color and color-difference meter. Results showed that both autoxidation and enzyme-catalyzed oxidation of brown-pigment precursors were responsible for the darkening of beet tissue. Histochemical studies on the localization of phenolase in red beets showed that the vascular tissue was the major site of enzyme activity. Apparently, the cell walls contained a small amount of phenolase. High phenolase activity was observed in both a beet extract and washed pulp of beets. Data are presented on the optimum pH and substrate specificity of beet phenolase. A solution containing 2% sodium carbonate and 5% sodium chloride was found to be a good solvent for extraction of the phenolase from washed beet pulp. At least four phenolic compounds were separated by paper chromatography.

INTRODUCTION

The darkening of sliced beets prior to canning has been recognized as a serious problem for many years. When beets are blanched insufficiently, the surface of beet slices, upon exposure to the air for a short time, acquire a brownish-red hue. Upon extended exposure, beet surfaces may appear black. This discoloration problem has been studied to some extent, but no clear-cut conclusions have been presented on the mechanism of darkening. Stevenson (1925) postulated that a polyphenoloxidase (tyrosinase) was the cause of darkening, and recommended heating beets to 66–82°C before cutting, and canning promptly. Clark and Moyer (1955) suggested that surface darkening may be linked with a heat-activated polyphenoloxidase and can be prevented by sufficient heat treatment of the beet tissue. These investigators proposed that the mechanism of darkening involved the oxidation of polyphenols to melanins through the formation of quinones with the aid of polyphenoloxidase, but no research work was presented to support this route of degradation.

The browning of sterilized intact and disrupted beet tissue has been found to occur in the presence of oxygen. When canned beet slices are exposed to the air for a short period, the surfaces darken (Lusas, 1956). Vilece *et al.* (1955), working with sterilized beet puree, found that a small amount of oxygen (6%) in the head space of a container was sufficient to cause browning near the surface. No studies have been reported on the identification of autoxidizable constituents in these products.

Some investigations (Tulliu, 1950; Wickberg, 1956; Wort and Shrimpton, 1959) have indicated the presence of phenolases in sugar beets. Several studies on the amino acid content of these roots (Bondiou, 1959; Mansford and Raper, 1954) have shown the presence of free and combined tyrosine. Wickberg (1956) found tyrosine and four or five other unidentified melanin-forming substances in sugar beets.

The present paper reports a study on the nature of the darkening of red beets, the localization and properties of beet phenolase(s), and the occurrence of phenolic compounds in beet tissue.

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MATERIALS AND METHODS

Beets. The beets used were the variety Rohnert Detroit Dark Red grown during the 1961 season near Madison, Wisconsin, and harvested in the middle of September. Beets with diameters of about 7.5–9 cm were selected and divided into 3 lots. Lot A was stored 1–6 months at 7°C, lot B was stored 1–6 months at −23°C, and lot C was canned. The canning procedure involved steaming the beets for 18 min at about 99°C, peeling and slicing (¼-inch slices), adding 1.5% NaCl to beet slices in cans, sealing, and heat processing for 35 min at 116°C. The canned beets were stored at 7°C for about 6 months. Fresh and frozen beets were peeled and cut with either an O'Brien Model H Dicer or a stainless-steel knife.

Washed beet pulp. Washed, nitrogen-treated, pigment-free beet pulp was prepared for studies on the properties of the phenolase. Fifty g of peeled, diced, frozen beets (lot B), with no visible brown tissue, were extracted with 200 ml of nitrogen-saturated water at room temperature (22°C). Extraction was carried out in a Waring blender at high speed for 5 min in an atmosphere of nitrogen to prevent oxidation of phenolic compounds. The slurry was centrifuged at 4,300 × G (at the center of the bottle) for 10 min, and the supernatant was decanted. The remaining pulp was extracted again with 200 ml of nitrogen-saturated water under the conditions mentioned above. The extraction procedure was repeated 4 more times. The yellowish-white material was freeze-dried in a glass laboratory lyophilizer. The dry pulp was pulverized in a Wiley laboratory mill with a 20-mesh screen. The resulting white powder was stored at −23°C.

Distilled water and C.P. chemicals were used. Mushroom tyrosinase was obtained from California Corporation for Biochemical Research, Los Angeles, California.

Instrumental and visual analyses. Color changes in beet purees were followed with a Hunter color and color-difference meter calibrated against a standard with the following Hunter values: $Rd = 6.4$, $a = +29.7$, and $b = +10.8$. For color measurements, the beet purees were placed in plastic dishes, each with a 6-cm diameter. Optical-density determinations were performed with a Bausch and Lomb Spectronic 20 colorimeter. The pH values were obtained with a Beckman Zeromatic pH meter. Microsections of beet tissue were cut with a freezing microtome (American Optical Co.). Photomicrographs were taken with Bausch and Lomb Model L photomicrographic equipment.

Darkening of beet tissue. Unheated beet puree was prepared by blending 140 g of frozen, diced beets (lot B) with 60 ml of nitrogen-saturated water (25°C) in a Waring blender for 3 min at high speed, followed by 2 min at low speed. During blending, the beet mixture was gassed with nitrogen. Unheated puree was used for the preparation of heat-treated and acid-treated purees. Heat-treated puree was prepared by heating 200 g of nitrogen-saturated beet puree for 60 min in an autoclave at 116°C. The top of the container was covered with an aluminum-foil cap. The heat-treated puree was cooled to 22°C in a nitrogen atmosphere before the color changes were measured. To study the darkening of puree at a low pH value, unheated beet puree was adjusted to pH 2.0 with 6*N* HCl solution. Canned beet puree was prepared from sliced beets (stored 6 months at about 7°C) by the procedure outlined above for unheated puree.

Ethanol-treated beet puree was prepared by blending, in an atmosphere of nitrogen, 100 g of frozen, diced beets (lot B) with 234 ml of absolute ethanol at 22°C. The final puree contained about 70% ethanol. A control puree was prepared by blending 100 g of frozen, diced beets with 234 ml of water.

To follow the color changes of untreated and treated beet purees in the presence of oxygen, each puree was transferred to a sample dish and pure oxygen was introduced into the agitated beet mass. The Hunter *Rd*, *a*, and *b* values were measured at short intervals until no significant changes were observed between consecutive readings. Nitrogen-saturated puree was used for determining the Hunter values at zero time.

Localization of phenolase. Cylindrical samples of beets (lot A) that had been in storage for about 5 months were obtained with a cork borer having an internal diameter of 13 mm. Sections with thicknesses of 120–150 μ were cut from partially thawed samples. Histochemical localization of tyrosinase in the tissue was accomplished by the method outlined by Gurr (1959). Brown pigments (melanins) are formed when infiltrated dopa is oxidized by the action of localized tyrosinase. The depth of the brown color is indicative of the enzyme concentration. Photomicrographs of beet sections were taken to illustrate the areas of browning.

Phenolase in aqueous beet extract. Beet extracts, which were used in studies of the activity and properties of phenolase in beets, were prepared by the following procedure. One hundred g of frozen, diced beets, with no visible brown tissue, were blended with 200 ml of nitrogen-saturated water in a Waring blender at high speed for 5 min. During blending, continuous nitrogen gassing was carried out. The homogenate was allowed to stand in a nitrogen atmosphere for 30 min and filtered under suction in a sintered-

glass filter. The filtrate was used immediately.

The phenolase activity in aqueous beet extracts (24°C) was measured by a modification of the method reported by Bailey *et al.* (1960). A volume of 100 ml of 0.05% dopa in 0.1*M* phosphate buffer solution (pH 6.8) was placed in a 250-ml beaker, and, while the dopa solution was stirred with a magnetic stirrer, 1 ml of aqueous beet extract was added with a fast-running pipette. The time at which 0.5 ml of beet extract had been added was taken as the zero time of the enzymic reaction. The optical density of the reaction solution (24°C) at 470 mμ was determined photometrically at short intervals. A 0.05% solution of dopa in 0.1*M* phosphate buffer (pH 6.8) was used as a blank. No significant change in optical density was observed during 60 min with a solution consisting of 1 ml of aqueous beet extract and 100 ml of phosphate buffer. The relative phenolase activity of the extract was expressed as increase in optical density per min.

The influence of pH on the activity of the beet phenolase was determined by a modification of the method of Ponting and Joslyn (1948). Determinations were made in 0.1*M* phosphate-citrate buffer solutions with pH values from 2 to 8 at 22°C. The procedure used was similar to the one already described for the determination of the phenolase activity except that 0.1% solutions of catechol were used instead of a solution of 0.05% dopa, which autoxidizes very rapidly in alkaline pH regions. Optical-density measurements of the catechol-buffer-beet extract solutions were taken at 470 mμ.

Studies on the substrate specificity of the beet phenolase were carried out with 14 different phenolic compounds. In general, solutions with 5% phenolic compounds were prepared, but saturated solutions of less soluble phenolic substances were used when necessary (Table 8). The original aqueous beet extract was diluted 20 times with 0.01*M* phosphate buffer (pH 6.8). One ml of either a 5% or saturated phenolic substrate solution was added to each tube with 10 ml of diluted beet extract. A 10-ml sample of diluted beet extract was used as a reference solution. All the tubes were incubated in a water bath for 12 hr at about 24°C. Thereafter, 1 ml of each solution was diluted with 10 ml of 0.01*M* phosphate buffer (pH 6.8), and optical density was measured at 470 mμ. A blank consisting of 1 ml of the particular substrate solution in 10 ml of 0.01*M* phosphate buffer (pH 6.8) was used in each case.

Phenolase in washed beet pulp. The activity of the phenolase remaining in washed, nitrogen-treated beet pulp was determined by the following procedure. Twenty ml of 0.01*M* phosphate buffer (pH 6.8) with 0.05% dopa were added to 0.5 g of lyophilized, washed, nitrogen-treated beet pulp powder in a plastic dish, 6 cm in diameter. The mixture was stirred during oxygen gassing, and the Hunter color values were recorded at short intervals. Used for zero time were the Hunter values of nitrogen-gassed slurry, consisting of 0.5 g of the pulp powder and 20 ml of 0.01*M* phosphate buffer (pH 6.8).

The substrate specificity of the phenolase remaining in the washed, nitrogen-treated beet pulp powder was studied. The concentration of each of 14 different compounds in 0.01*M* phosphate buffer (pH 6.8) was 0.05%. In each spot plate cell, 0.5 ml of a particular phenolic solution was mixed with 10 mg of lyophilized, washed beet powder. The mixtures were held for 2 hr at about 22°C in a water-saturated chamber, and the relative action of the enzyme in the beet pulp on the various phenolic compounds was determined on the basis of the color developed. The intensity of the color for each beet pulp mixture was recorded according to the following designations: 0, no color development; 1, slight reddish-yellow; 2, moderately reddish-yellow; 3, yellowish-brown; and 4, black. To compare the specificity of the phenolase of the beet pulp with the specificity of mushroom tyrosinase, similar experimentation was carried out with 0.1 ml of a solution of mushroom tyrosinase containing 0.01 mg of the enzyme.

For extraction of the phenolase from the lyophilized beet pulp powder, a solution containing 2% sodium carbonate and 5% sodium chloride was used exclusively. Fifty mg of the lyophilized washed pulp were extracted with 10 ml of solvent by shaking the mixture for 20 min in a stoppered 13.5-ml plastic centrifuge tube in the presence of a few small glass beads. Shaking was done with a Burrell wrist-action shaker. The suspension of pulp was then centrifuged 5 min in a Spinco Model L ultracentrifuge using a rotor No. 40 at 6,590 × G. The supernatant was decanted and filtered in a sintered-glass filter under reduced pressure. The residue from the first extraction was re-extracted 4 more times by the same procedure except that extraction time was reduced to 10 min and the amount of solvent used in each extraction was only 9 ml. Enzyme activity for each extract was determined by the method previously outlined for the measurement of phenolase activity in aqueous beet extracts. These determinations were made immediately after the enzyme extraction because it was observed that the extracts became cloudy upon standing.

Phenolic compounds in red beets. One hundred g of frozen, diced beets (lot B) were blended with 300 ml of 95% ethyl alcohol containing 1% HCl in a Waring blender at high speed for 5 min in an atmosphere of nitrogen. The homogenate

was centrifuged for 10 min at about 4,300 × G (at the center of the bottle), and the supernatant was decanted. The residue in each centrifuge bottle was extracted again with 300 ml of the solvent. The combined centrifugates were concentrated to about 25 ml under vacuum in a rotary evaporator with the flask in a water bath at about 40°C. This extract was filtered through Whatman No. 42 filter paper.

The method of Rudkin and Nelson (1947) was not suitable for purification of the phenolic beet extract, since most beet pigments were also precipitated by lead acetate. A method somewhat similar to that used by Moore *et al.* (1958) was found to give better results. The method is based on retention of the phenolic compounds, particularly phenolic amino acids, in a column of a cationic ion-exchange resin. A column was prepared of Dowex 50W-X8 (200–400-mesh), 25 cm long and 1.5 cm in diameter. Before the column was packed, the resin was treated as recommended by Moore and Stein (1951). Before separation was started, the column was rinsed with 0.1*M* citrate-phosphate buffer solution (pH 3.4).

The pH of the concentrated phenolic extract of beets was adjusted to 3.0 with 4*N* NaOH solution. Two ml of this extract were pipetted onto the resin column, and development was started with 0.1*M* citrate-phosphate buffer (pH 3.4) under atmospheric pressure and at room temperature (22°C) until pigment was no longer observed in the effluent. Final elution was achieved with 0.1*M* citrate-phosphate buffer with a pH of 6.8. Elution was carried out until the eluate gave a negative reaction for phenolic compounds with Folin-Denis reagent (Rosenblat and Peluso, 1941). The total eluate obtained (after the change of eluant buffer from pH 3.4 to pH 6.8) was concentrated from about 70 ml to approximately 8 ml, under vacuum at about 40°C. The final phenolic extract was adjusted to pH 3 with 2*N* HCl solution and stored at 7°C until used.

The phenolic compounds in the concentrated extract were separated by paper chromatography on Whatman No. 1 filter paper with butanol–acetic acid–water (4:1:5) used as the developing solvent. Standard solutions of tannic acid, chlorogenic acid, caffeic acid, L-3,4 dopa, tyrosine, catechol, and D-catechin were run concurrently with the phenolic extract. Samples of about 10–30 ml of the beet extract were applied to the paper. The papers with the applied spots were allowed to equilibrate with the solvent system for 6 hr, and then were developed by the ascending technique at room temperature (22°C). After a developing time of 16 hr, the chromatograms were dried and sprayed with modified Folin-Denis Reagent (Johnson and Schaal, 1952) and exposed in a glass jar for about 30 min to the fumes from concentrated ammonium hydroxide. For the detection of amino acids on developed paper chromatograms, 0.2% ninhydrin solution in 95% ethyl alcohol was sprayed on the paper. The R_f values of the known and unknown spots were calculated from measurements made to the center of the spots.

RESULTS AND DISCUSSION

Color changes of disrupted beet tissue. Beet puree was used to provide uniform distribution of oxygen and brown-pigment precursors and to produce pronounced color changes. Preliminary studies with beet puree indicated that no visual color changes were apparent in nitrogen-treated puree (approx pH 6.2) whereas darkening was observed in oxygen-treated puree after a short incubation period at room temperature. Experimentation was carried out to obtain more information on the oxidative reactions involved in the darkening of beet puree.

The Hunter color values of unheated beet puree (approx pH 6.2) in the presence of oxygen at various holding times are recorded in Table 1. During the first 6 min, the Hunter color values of the beet puree decreased rapidly due to the formation of oxidized, colored constituents. For a holding period of 35 min, ΔRd, Δa, and Δb were respectively −0.8, −23.5 and −4.3. The decrease in the a/b value from 5.1 to 3.4 may be interpreted as a shift in the hue of beet puree from red toward yellow.

Some investigations have indicated that the darkening of beet tissue in the presence of oxygen is due to the action of oxidases on brown-pigment precursors (Clark and Moyer, 1955; Wickberg, 1956). To obtain more information on the role of phenolase on the oxidative browning, an investigation was conducted on the changes of beet puree, treated separately with heat and ethanol to inactivate the oxidizing enzymes. The color changes of heated puree in the presence of oxygen are presented in Table 1. Apparently the a/b value of heated puree did not change significantly with oxygen treatment, although both the a and b values respectively decreased 4.5 and 1.8 units during 70 min of holding time. Autoxidation of brown-pigment precursors can be linked

Table 1. Color changes in beet tissue at 22°C in the presence of oxygen.

Time (min.)	Hunter color values			
	Rd	*+a*	*+b*	*a/b*
Unheated puree				
0	1.4	26.9	5.3	5.1
2	1.3	19.8	4.0	5.0
4	1.2	12.8	2.6	4.9
6	1.1	9.8	2.2	4.5
10	1.0	7.1	1.7	4.2
20	0.7	4.4	1.3	3.4
35	0.6	3.4	1.0	3.4
140	0.6	2.8	0.8	3.5
Heated puree				
0	3.3	17.2	7.7	2.2
2	3.2	15.6	7.0	2.2
4	3.1	14.5	6.8	2.1
6	3.1	14.3	6.8	2.1
10	3.0	13.9	6.4	2.2
20	3.0	13.4	6.3	2.1
70	2.9	12.7	5.9	2.2
Puree without alcohol treatment				
0	2.8	15.0	6.0	2.5
2	2.7	10.9	5.4	2.0
4	2.7	10.6	5.3	2.0
6	2.7	10.3	5.2	2.0
10	2.7	9.8	5.1	1.9
20	2.7	9.0	5.0	1.8
Alcohol-treated puree				
0	3.0	16.4	6.2	2.6
2	3.0	14.2	6.0	2.4
4	2.9	14.0	5.9	2.4
6	2.9	13.8	5.8	2.4
10	2.9	13.6	5.7	2.4
20	2.8	12.9	5.6	2.3
80	2.8	11.6	5.4	2.2
Acidified puree (pH 2.0)				
0	3.5	23.0	6.5	3.5
2	3.3	20.8	6.3	3.3
4	3.2	19.9	6.2	3.2
6	3.2	19.4	6.2	3.1
10	3.1	18.6	6.1	3.1
20	3.0	17.9	5.9	3.0
35	3.0	17.4	5.7	3.1
Canned beet puree				
0	4.5	33.2	7.9	4.2
2	4.0	29.5	6.8	4.3
4	3.9	27.9	6.8	4.1
8	3.8	26.6	6.6	4.0
20	3.6	25.3	6.4	4.0
100	3.5	21.6	6.2	3.5
140	3.5	20.3	6.1	3.4

TABLE 1 (concluded)

Time (min.)	Hunter color values			
	Rd	*+a*	*+b*	*a/b*
Washed pulp slurry with dopa				
0	19.8	3.0	17.8	0.2
1	12.3	15.7	17.9	0.9
3	7.9	22.2	17.6	1.3
6	6.2	23.3	17.0	1.3
10	4.5	21.5	16.8	1.3
20	2.0	13.5	8.2	1.6
28	1.2	8.9	5.3	1.7
40	0.9	6.4	3.8	1.7

with the darkening of heated beet puree. Ethanol was used in an attempt to inactivate oxidizing enzymes prior to oxygen treatment of the puree without significantly altering the structure of pigments and other heat-sensitive compounds. As shown in Table 1, the Hunter color values for both ethanol-treated and untreated purees changed in the presence of oxygen during short holding periods. However, changes in the Hunter color values were not as marked in ethanol-treated puree as in the control puree. The Hunter *a* values decreased markedly during oxygen treatment of both purees. The Δa values for ethanol-treated and untreated purees from the same batch were respectively −4.8 and −7.3 during a holding period of 80 min. These observations suggest that both autoxidizable and enzyme-oxidizable compounds are present in the beet tissue.

The Hunter color values of an acid-treated beet puree (pH 2.0) at various holding periods during oxygen gassing are presented in Table 1. During the first 6 min, the Hunter values changed rapidly. The ΔRd, Δa and Δb values for a holding time of 35 min were respectively −0.5, −5.6, and −0.8. These changes, however, were not as marked as those of unheated beet pulp at about pH 6.2. At a pH level of 2, phenolase activity would be negligible. Consequently, the autoxidation of brown-pigment precursors apparently accounts for the color changes in the acidified beet puree. The shift in hue of the puree from red to yellow is apparent again from the *a/b* values.

To determine whether autoxidizable brown-pigment precursors were present in

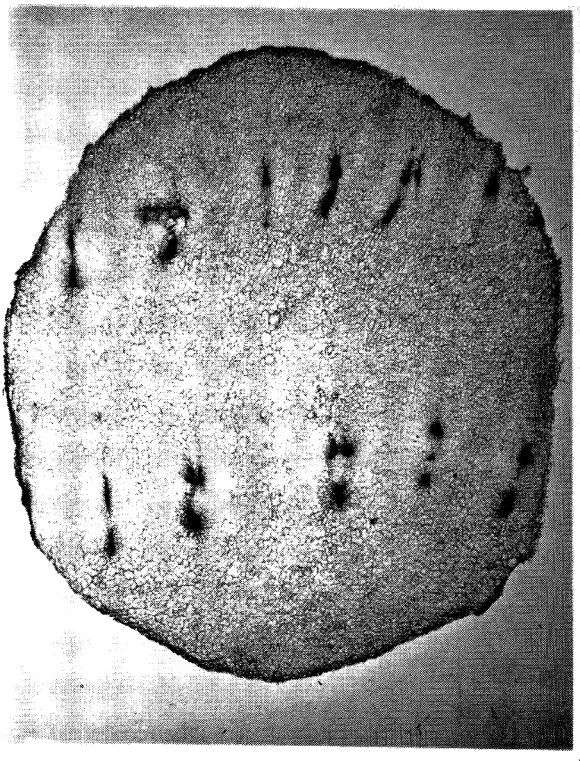

Fig. 1. Photomicrograph of a transverse section of fresh beet tissue after treatment with dopa. (×14).

Fig. 2. Photomicrograph of a longitudinal section of fresh beet tissue after treatment with dopa. (×14).

canned beets, Hunter color values were obtained with puree saturated with oxygen. As shown in Table 1, the Hunter color values of the puree decreased rapidly during the first 2 min, and decreased more slowly during the next 138 min. Again the *a* value was changed most rapidly during the holding time. Moreover, the respective *a/b* values of 4.2 and 3.3 for holding times of 0 and 140 min indicate that during oxidation, the hue of the puree was shifting from red to yellow. Autoxidation of brown-pigment precursors in this puree was presumably responsible for the hue shift.

Phenolase in red beet tissue. Vascular bundles in transverse and longitudinal beet sections treated with dopa became highly pigmented (Figs. 1, 2). Consequently, the vascular tissue may be considered as the major site of phenolase. The cell walls contained some phenolase since they darkened slightly with the dopa treatment. Wickberg (1956), who investigated the non-pigmented *Beta vulgaris* (commonly called sugar beets), observed high phenolase activity along the vascular bundles, inside the epidermis, and in the sprouts. Other investigators have reported distribution of phenolases in the vascular tissue of grapes (Hussein and Cruess, 1941), apricots (Samish, 1935), and pears (Christ and Batier, 1931). In this study, consideration has been given to the phenolase activity in an aqueous extract of beet tissue and in washed, pigment-free beet pulp. Fig. 3 shows the rate of color formation in the beet extract-dopa reaction solution at 23°C. The increase in optical density was rapid during the first 15 min after dopa addition, and slower thereafter. The enzyme activity (ΔO.D./min), calculated from the slope of the straight line, was 0.018.

The high concentration of phenolase in the washed beet pulp is illustrated in Table 1 by the changes in Hunter color values of pulp slurry with added dopa. The *Rd* value

of the dopa-treated slurry decreased rapidly, from 19.8 to 7.9, within a 3-min holding period. On the other hand, the *b* value decreased progressively over a period of 40 min. During the first 6 min of reaction time, the Hunter *a* value increased 20.3 units, and then decreased 16.9 units within the next 34 min. The *a*/*b* value increased 1.1 units during the first 6 min of holding time. This increase in the *a*/*b* value could be attributed to the formation of a red pigment, dopachrome, in the presence of a high concentration of active phenolase in the washed, non-oxidized pulp.

Table 2. Extraction of phenolase from washed, nitrogen-treated beet pulp with a solution containing 2% sodium carbonate and 5% sodium chloride.

Extraction no.	Optical density of extract (280 mμ)	Phenolase activity of extract (ΔO.D./min)
1	0.485	5×10^{-3}
2	0.087	3×10^{-4}
3	0.058	3×10^{-5}
4	0.040	....
5	0.025	

Table 2 shows that a large amount of enzyme was removed with a Na_2CO_3–NaCl solution during the first extraction of beet pulp, and that smaller amounts were solubilized during the second and third extractions. Further extractions did not provide significant amounts of active enzyme. A white haziness was formed in the first 3 enzyme extracts (pH 11.5) during standing for 1 hr at room temperature. Wickberg (1956) observed that during the purification and storage of sugar beet phenolases in pH 6.8 phosphate buffer, the enzymes aggregated to form precipitates with high phenolase activity.

The influence of pH on the activity of crude phenolase in an aqueous beet extract is illustrated in Fig. 4. The optimum pH of the crude phenolase in citrate-phosphate buffer was between 6.5 and 7.0 with catechol as the substrate. The activity of the enzyme was totally inhibited at pH 2.0.

Table 3 shows the action of the phenolase in an aqueous extract of beets on 14 different phenolic compounds representing monophenols, ortho-, meta-, and para-diphenols.

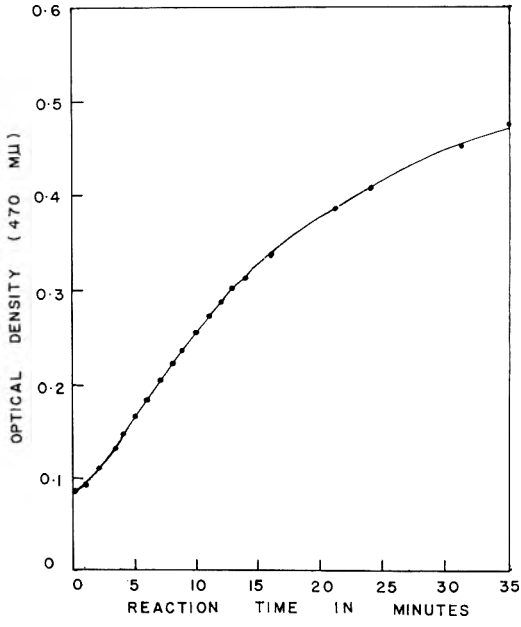

Fig. 3. Rate of color formation in a dopa solution (23°C) with beet phenolase.

Dopa, catechol, and other ortho-dihydroxy phenolic compounds were oxidized rapidly by the action of the beet phenolase. Tyro-

Table 3. Substrate specificity of phenolase in beet extract and washed beet pulp.

	Relative activity of phenolase		
	Beet extract	Beet pulp	Mushroom
Substrate	ΔO.D./12 hr	Degree of color development	Degree of color development
L-3,4 dopa	0.240	4	4
L-tyrosine [a]	0.078	4	4
Chlorogenic acid	0.037	1	1
Caffeic acid [a]	0.070	2	3
Tannic acid	0.025	1	1
Quinic acid	....	0	0
Ellagic acid [a]	....	0	0
D-catechin [a]	0.095	3	3
Catechol	0.085	3	3
Resorcinol	....	0	0
Hydroquinone	0.017	1	1
Phloroglucinol	0.010	1	1
Pyrogallol	0.220	3	2
Guaiacol	....	0	0
O-phenylene diamine	....	0	0

[a] The following phenolic compounds were not soluble to the extent of 5% and in these cases, saturated solutions were used: L-tyrosine, caffeic acid, ellagic acid and D-catechin.

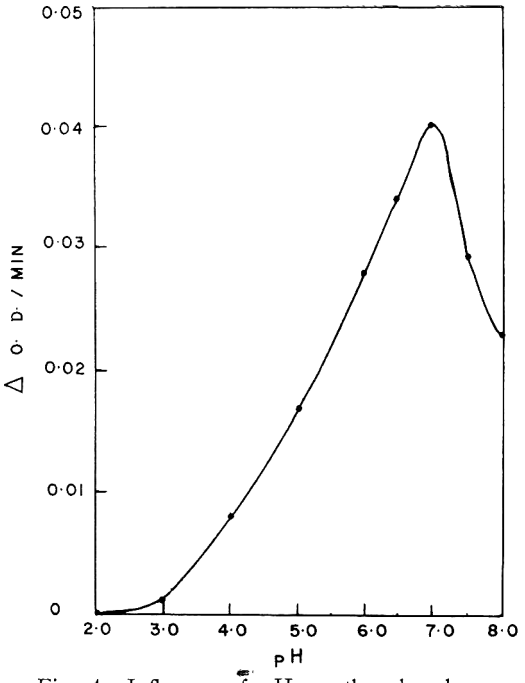

Fig. 4. Influence of pH on the phenolase activity.

sine, a monohydroxy compound, and pyrocatechol were also oxidized rapidly. The catalytic effect of phenolase on the oxidation of resorcinol, representing meta-dihydroxy compounds, was not exhibited. Hydroquinone (para-dihydroxy compound) was oxidized slightly by the beet phenolase. Phloroglucinol, guaiacol, and orthophenylene diamine were not oxidized appreciably by the beet enzyme. Table 3 also shows the effect on these phenolic compounds of the phenolase in washed, pigment-free beet pulp. The data indicate that the phenolase remaining in the washed, non-oxidized beet pulp has the same substrate specificity as the phenolase of the aqueous extract. These results in Table 3 indicate a similarity between the substrate specificity of beet phenolase and mushroom tyrosinase.

Phenolic compounds in red beets. Experimentation was carried out in order to identify the phenolic compounds present in beets. The concentrated phenolic extracts contained high concentrations of pigments and sugars, which interfered in paper chromatographic separation of phenolic compounds. Therefore, an attempt was made to remove most of the interfering substances by ion-exchange chromatography. Preliminary experiments showed that a column of cationic ion-exchange resin (Dowex 50W-X8), adjusted to pH 3.4, adsorbed tyrosine and chlorogenic acid from a solution of these compounds in 0.1*M* citrate-phosphate buffer (pH 3.4). It was also observed that most beet pigments and sugars passed rapidly through the same column upon washing with the pH 3.4 buffer. On the basis of these observations, a sample of the original concentrated phenolic extract of beets was placed on a column of the cationic exchange resin adjusted to a pH of 3.4. As expected, most beet pigments and sugars were removed easily by the pH 3.4 buffer. Very slight coloration was produced when eluate fractions were treated with Folin-Denis reagent. Thus, most phenolic compounds were retained in the column. When visible pigment was no longer present in the eluate, the phenolic compounds in the column were eluted with a buffer of pH 6.8 and pronounced coloration was noted in the eluate fractions with added Folin-Denis reagent.

A paper chromatographic technique was used to separate the partially purified, concentrated extract of beets. At least 4 compounds were separated, as shown by the appearance of blue spots on developed chromatograms sprayed with modified Folin-Denis reagent. Results given in Table 4 show the R_f values of these unknown spots and those of standard phenolic compounds, which were chromatographed concurrently with the beet extract for the purposes of identification. The R_f values of the unknown spots 2 (R_f = 0.24) and 3 (R_f = 0.46)

Table 4. R_f values of phenolic compounds in a beet extract.

Unknown compounds	R_f	Phenolic compounds	R_f
1	0.10 (?)		
2	0.25 (Dopa)	L-3,4 dopa	0.24
3	0.46 (Tyrosine)	L-tyrosine	0.46
4	0.55 (?)		
		Chlorogenic acid	0.63
		Tannic acid	0.66
		D-catechin	0.67
		Caffeic acid	0.86
		Catechol	0.93

respectively corresponded with those of standard spots for dopa and tyrosine. The unknown spots 1 and 4 were not identified. Chromatograms, sprayed with 0.2% ninhydrin solution, showed 5 amino acid spots including 2 with R_f values of 0.24 and 0.47. These R_f values respectively corresponded to those of dopa and tyrosine. Wickberg (1956) reported that tyrosine plays an important role in the darkening process of sugar beets and that at least 4 or 5 other phenolic compounds take part in this reaction.

The results obtained in this work seem to indicate that dopa and tyrosine, along with at least 2 other unidentified phenolic compounds, are present in red beets. Dopa and tyrosine probably play an important role in the darkening of red beets. The number and identity of the other phenolic compounds present in beets, as well as their role in the browning reaction, should be investigated.

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Amino Acids in Fermented Bread Doughs [a]

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(Manuscript received July 26, 1962)

SUMMARY

Nineteen free amino acids were identified and quantified in the dialyzable fraction from fermented bread doughs ready for baking. Amino acids account for the majority of the dialyzable nonvolatile amino compounds in this fraction. Nine of the amino acids found in bread doughs were also identified in a preferment brew. Peptides did not appear in the dialyzable extracts, and are assumed to be absent, or present in very low concentrations, in the doughs. Most of the amino nitrogen of fermented doughs remains in the nondialyzable fraction. The amino acid fraction, soluble-protein fraction, and volatile nitrogen compounds, measured as ammonia, make minor contributions to the total amino content of bread doughs.

INTRODUCTION

Crust browning is essential for the production of bread with an acceptable flavor and aroma. It has been hypothesized that crust browning is due to a Maillard-type reaction, rather than caramelization or pyrolysis of sugar and starch. Several publications have described the volatile products formed from the reactions of sugars and amino acids. Akabori (1933) isolated and characterized the aldehydes from the reaction of several amino acids and sugars. Rothe (1960) has shown that separate additions of several amino acids to rye bread doughs resulted in an increase of the corresponding aldehydes, formed from the amino acid with loss of one carbon atom and an amino group. The same products were obtained in test-tube experiments using the same amino acids and xylose. Kiely *et al.* (1960) investigated the aromas from the reaction of twenty amino acids and eight sugars. The reaction products of leucine, histidine, and arginine with glucose were described as being bread-like. In a recent publication, Hodge and Nelson (1961) described the preparation of galactosylisomaltol and isomaltol from lactose and an amine. They postulated that these compounds may exist in bread, and incorporated the compounds into doughs for organoleptic evaluation after baking (Hodge and Moser, 1961).

Since doughs appear not to have been analyzed for amino compounds, which are potential Maillard-type-reaction precursors, it was felt that a systematic approach to the problem of flavor in bread requires investigation of such amino compounds. While our work was in progress, a publication (Kretovich and Ponomareva, 1961) describing the free amino acid content of wheat flour, rye flour, proofed rye dough, rye bread, and white bread appeared. According to Kretovich, a decrease in free amino acids occurs in crust during the baking of proofed rye doughs.

EXPERIMENTAL

Sample preparation. Proofed sponge doughs were prepared by a modification of a standard test formula (Anon., 1957) modified by using 49 g of sucrose instead of 35 g and by incorporating 21 g of dry milk solids into the doughs. A commercial baker's patent flour, analyzing 0.43% ash and 12.70% protein (14% moisture basis), was used.

For amino acid separations, 100-g samples of proofed doughs were suspended in 100-ml portions of 0.1*M* acetic acid by blending, transferred

[a] Presented at the 22nd Annual Meeting of the Institute of Food Technologists, June, 1962, Miami Beach, Florida.

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

to dialysis bags, and dialyzed overnight against distilled water (about 1 L) at 4°C. Each dialysate was passed through a column containing 40 g of Dowex 50-X4 (H^+ form). The column was washed with approximately 1 L of distilled water and eluted with 5*M* ammonium hydroxide. Eluate (250 ml) was collected and concentrated to near dryness with a rotary evaporator under reduced pressure, then transferred to a 5-ml beaker with small portions of distilled water, and evaporated to dryness, over a 3-day period, in a vacuum desiccator over phosphorus pentoxide at approximately 10–20 mm Hg. The solid material was diluted to 2 ml with 0.1*M* hydrochloric acid.

Soluble proteins were extracted from 50-g samples of proofed dough by the procedure of Pence *et al.* (1954). The protein solutions (200-ml volumes) were placed in dialysis bags and dialyzed against tap water overnight. The final volumes were approximately 215 ml.

For ammonia determinations, 25-g samples of proofed doughs were lyophilized for 40 hr, ground in a mortar to a fine powder, equilibrated with water overnight in a closed container, and then lyophilized for a second 40-hr period. Ten-g samples of the lyophilized dough were suspended in 30–40 ml of 0.1*M* acetic acid, placed in dialysis bags, and dialyzed against 93-ml volumes of distilled water for 1½ days at 4°C. The final volumes of the dialysates were approximately 88 ml.

Pre-ferment brews were prepared to Swortfiguer's (1955) formula and fermented for 6 hr at 35°C. One liter of pre-ferment was passed through 230 g of Dowex 50 (H^+ form). The column was washed with 5 L of distilled water and eluted with 1*M* ammonium hydroxide. The eluate, approximately 2 L, was treated as described for the separation of amino acids from proofed doughs.

Analytical methods. To obtain the total amino acid content, 0.1 ml of the solution of amino acid fraction from proofed doughs was diluted 1000-fold with glass-distilled water, and 1-ml portions used for each analysis. The quantitative ninhydrin reagent was prepared according to the procedure of Yemm and Cocking (1955). A calibrated curve was constructed from the optical densities at 570 mμ for various concentrations of arginine hydrochloride, and, from this curve, the concentration of the amino acid fraction was obtained in terms of micromoles of arginine per ml.

The two-dimensional ascending paper-chromatography technique of Redfield (1953) was used for resolving the amino acid fractions from proofed doughs and pre-ferments. Each sample, 4.5–7 μl, was applied as a spot 2 cm from each edge of a 9×9-cm sheet of Schleicher & Schuell no. 507 filter paper. Chromatograms containing known amino acids were run in the same jar with the unknown. The papers were dipped in 0.1% ninhydrin in water-saturated butanol and air-dried overnight to develop the amino acid spots.

Quantitative analysis of the individual amino acids in the dialyzable material from proofed dough was performed according to the procedure of Spackman *et al.* (1958), in a Beckman-Spinco Model 120 amino acid analyzer.

For the analysis for peptides, 0.2-ml volumes of the amino acid fraction from proofed doughs were pipetted into small Pyrex test tubes, and approximately an equal volume of concentrated hydrochloric acid was added. The tubes were sealed, heated 22 hr at 110°C, and opened, and the contents filtered through a fine-sintered-glass funnel. The solutions were evaporated over sodium hydroxide pellets in a vacuum desiccator at 10–20 mm Hg, and each was diluted to 0.2 ml with 0.1*M* hydrochloric acid. Samples (4.5–7 μl) were spotted on the papers and chromatographed by the technique of Redfield (1953).

Ammonia was determined by the Nessler test. To avoid an interfering pink color that developed when the Nessler test was applied to the dialyzable material, the sample was first purified by the diffusion procedure of Rose (1950). The technique was modified as follows: 5 ml of the dialyzable material was placed in one part of the dish, the central portion of the ground-glass cover was wetted with 0.1 ml of 20% sulfuric acid, and 0.5 ml of 20% aqueous sodium hydroxide was placed in another part of the dish. The cover was placed on the dish, the base and dialyzable material were mixed, and the diffusion was allowed to proceed for 3–4½ hr. Ammonia was determined by Nesslerization with a commercial reagent (Regent Nessler Compound, Regent Scientific Co., 919 Brook Ave., New York, N. Y.). The diffusate was diluted to 10 ml, a 4-ml portion was withdrawn and diluted to 10 ml with distilled water, and 0.5 ml of the Nessler reagent was added. After 20 min, optical density was read at 435 mμ. A blank prepared by substituting distilled water for the dialysate was subjected to the diffusion technique, and a 4-ml portion was diluted and Nesslerized as described.

Amino nitrogen was determined by the Van Slyke procedure on soluble protein fractions and lyophilized dough.

RESULTS AND DISCUSSION

Twenty amino acids were tentatively identified in the dialyzable fraction from proofed doughs. Most of these were determined by use of both paper chromatography and ion-exchange chromatography (Table

Table 1. Free amino acids in dialysate from proofed bread dough.

Amino acid	Paper chromatography				Ion-exchange chromatography
	Solvent A[a] (R_f)		Solvent B[b] (R_f)		
	Dialysate	Standard	Dialysate	Standard	Dough (μM/100 g)
Alanine	0.50	0.49	0.34	0.32	9.22
Arginine	0.06	0.06	0.11	0.10	3.70
Aspartic acid	0.31	0.33	0.09	0.09	2.53
Cystine	....	....	....	....	0.98
Cysteic acid	....	....	....	....	1.85
Glutamic acid	0.45	0.44	0.11	0.12	16.03
Glutamine	0.35	0.34	0.19	0.18	....
Glycine	0.34	0.33	0.22	0.16	5.07
Histidine	0.24	0.21	0.29	0.26	0.50
Isoleucine	0.67	0.67	0.69	0.71	1.94
Leucine	0.65	0.61	0.87	0.88	5.04
Lysine	0.16	0.15	0.12	0.10	4.0
Methionine	0.53	0.54	0.56	0.57	1.68
Phenylalanine	0.62	0.61	0.69	0.70	4.27
Proline	0.62	0.64	0.27	0.27	9.60
Serine	0.41	0.37	0.37	0.36	1.04
Threonine	0.46	0.45	0.65	0.68	2.40
Tryptophan	0.37	0.35	0.67	0.68	0.99
Tyrosine	0.48	0.50	0.36	0.36	4.22
Valine	0.59	0.57	0.47	0.45	5.12

[a] Solvent A, methanol–water–pyridine (80/20/4).
[b] Solvent B, t-butyl alcohol–methyl ethyl ketone–water–diethylamine (40/40/20/4).

1). Cystine and cysteic acid were tentatively identified solely on the basis of their elution volumes obtained from ion-exchange chromatography. Cystine was not separated from lysine in the paper chromatographic technique employed. Glutamine was not quantified, since the ion exchange chromatography was performed under conditions that did not resolve it.

Sullivan and Payne (1951) found essentially the same amino acids in flour, except for cystine, tryptophan, and cysteic acid, which were detected in the present study. (The cysteic acid may have been an artifact formed during isolation of the amino acid fraction from proofed doughs.) Sullivan reported asparagine, which was not found in the present study.

Kretovich and Ponomareva (1961) found, in studies of wheat flour and baked bread, essentially the same amino acids found in the present work, plus γ-aminobutyric acid and asparagine. They determined several of these amino acids quantitatively. It is difficult to compare their results with ours, because they did not analyze proofed doughs prepared from white flours. In addition, comparisons are complicated by the varietal differences of the wheats, unknown history of the grain and flour samples, and the different analytical techniques employed.

Table 1 shows the concentrations of 19 of the amino acids identified in the dialyzable fraction of doughs. Eleven minor components were eluted from the ion-exchange columns during analysis of the amino acid fraction. No attempt was made to identify these, however, because of their low concentration or because of the lack of known reference compounds.

The question of the origin of the various amino acids in proofed doughs is complicated by many factors. The action of numerous flour and yeast enzymes on compounds present in proofed doughs is not well understood. Two potential sources of the free amino acids are synthesis of amino acids from a nitrogen source by yeast, and proteolytic activity of wheat enzymes on proteins. A pre-ferment brew was analyzed for free amino acids, because this system allows study of the action of yeast under somewhat controlled conditions, without the presence of flour and its proteolytic en-

Table 2. Amino nitrogen content of various fractions from proofed dough.

	Amino nitrogen (mg/100 g dough)	% of total amino nitrogen in dough
Dialysate of proofed doughs [a]	1.6	2.7
Water-soluble protein fraction [b]	2.0	3.4
Ammonia [c]	0.39–0.89	0.6–1.5
Proofed doughs (lyophilized) [b]	58.6	100

[a] Calculated from ninhydrin determinations.
[b] Obtained from Van Slyke amino nitrogen analyses.
[c] Volatile bases determined as ammonia by Nessler's reagent.
Duplicate samples were obtained from a proofed dough.

zymes. The same principal amino acids found in dough dialysates were present in the pre-ferment brew: these are arginine, aspartic acid, glutamic acid, glutamine, glycine, isoleucine, leucine, proline, and valine. Their presence in the brew indicates that the action of proteolytic flour enzymes on proteins present in dough is not the sole source of amino acids.

Portions of the amino acid fraction from proofed doughs were hydrolyzed, and chromatograms were obtained on both the hydrolyzed and unhydrolyzed material. No significant differences in position, size, and intensity of the spots were noted in the two fractions. This similarity in the chromatograms indicates the absence of peptides in the amino acid fraction, or their presence only in low amounts.

Water-soluble proteins, free amino acid fraction, and volatile bases measured as ammonia account for less than 10% of the total amino nitrogen in proofed doughs (Table 2). Most of the amino nitrogen in fermented doughs appears to be in the water-insoluble fraction.

This finding suggests that the insoluble proteins should be assessed to determine their importance in crust browning and flavor production.

ACKNOWLEDGMENT

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

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Kinetics and Energetics of Thermal Inactivation and the Regeneration Rates of a Peroxidase System [a,b]

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(Manuscript received April 22, 1962)

SUMMARY

The kinetics of thermal inactivation of peroxidase were found to he first-order for the range of temperatures 85–100°C. The experimental activation energy was 25.1 kilocalories per mole; free energy change, 27.0–27.5 kilocalories per mole; and entropy change, a negative 7.39–7.89 calories per mole degree. This indicated that the transfer of energy was extremely slow or the activated molecule was actually more rigid than the native molecule, the latter being more likely than the former. No regeneration occurred when peroxidase had been completely inactivated.

Regeneration was found to involve a lag period of approximately 20 hr, a rapid rate period of 1–2 days, a point of maximum regeneration at 2–10 days, and finally a decrease in activity after inactivation.

The thermal destruction time (z value) of 49.8°F indicated that in high-temperature short-time processes the criterion for sufficiency might necessarily be based on the time required to inactivate peroxidase rather than on the time required to sterilize the product.

Peroxidase reacts with hydrogen peroxide to produce an activated complex that can oxidize a wide range of donor molecules such as polyphenols and amino phenols. These reactions are undesirable in processed food material, because they effect a change in the non-volatile flavoring components (Farkas *et al.*, 1956; Guyer and Holmquist, 1954; Nebesky *et al.*, 1950). Thus it is important to either totally or partially inactivate the enzyme in any food material where its active concentration is sufficiently high to be detrimental.

With the advent of high-temperature short-time sterilization (HTST), peroxidase has received attention primarily because of its high thermal-death-time coefficient (z value) and apparent regeneration under HTST conditions (Farkas *et al.*, 1956; Guyer and Holmquist, 1954; Zoueil and Esselen, 1959). It appears that the advantages of HTST processing resulting from the rapid inactivation of bacteria by high temperatures are offset to a considerable extent by the relatively lower rate of peroxidase destruction at high temperatures.

Thermal inactivation and regeneration of peroxidase in plant material has been investigated; however, no attempt has been made to determine the nature of inactivation based on the energetics that might explain the mechanisms of the regeneration process.

This study was made to investigate thermal inactivation of a pure peroxidase preparation with particular reference to the kinetics and energetics of the process and to suggest an explanation for regeneration.

EXPERIMENTAL METHODS

Assay for peroxidase activity. The guaiacol test used by George (1953) was modified to improve its range, sensitivity, and accuracy. The modified reaction mixture consisted of 2 ml of a 10m*M*

[a] Taken in part from a Ph.D. thesis, Rutgers, The State University.

[b] A paper of the Journal Series, N. J. Agricultural Experiment Station, Rutgers, The State University, Department of Food Science, New Brunswick, N. J.

[c] Current address: Procter and Gamble Co., Winton Hill, Technical Center, Cincinnati 24, Ohio.

solution of guaiacol in water, 1 ml of enzyme solution, and 20 μl of 15μM hydrogen peroxide. The H_2O_2 was placed in the sample cell of a Beckman DK2 spectrophotometer modified for direct recording of absorbance at 470 mμ vs. time. The enzyme-guaiacol mixture was added to the sample cell with an automatic pipetting syringe and the curve of absorbance vs. time was recorded.

Heating conditions. The procedure described above, termed assay of guaiacol oxidation rate (GOR), was used to develop a standard curve of activity vs. peroxidase concentration (Fig. 1). The enzyme used was a highly purified (R.Z. = 3.02) preparation of horseradish peroxidase obtained from Worthington Biochemical Corp., Freehold, N. J.

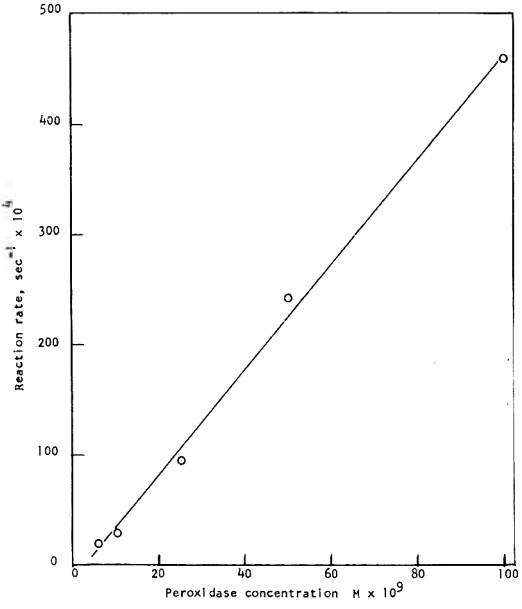

Fig. 1. Molar activity of peroxidase preparation E_2 in water.

A stock solution of $5 \times 10^{-7}M$ peroxidase in 10mM phosphate buffer, pH 7, was prepared fresh for each experiment. Pyrex glass tubes (3 mm outside diameter, 0.6 mm wall thickness, 20 cm length) were filled with stock solution to within 2 cm of both ends, sealed by fusion and placed in a 20-tube position holder in preparation for heating.

Heating was carried out in a constant-temperature (±0.2°C) oil bath. The tube-containing holder was placed in the bath and agitated by hand until the tube contents reached heating-medium temperature. After heating, the tubes were cooled in detergent solution, washed, dried, and assayed for peroxidase activity. Heat penetration tests were run with each heating experiment to determine the lag correction factors. These factors were subtracted from the total heating times to give the corrected times at each temperature.

Inactivation and regeneration conditions. To study the effects of degree of thermal process on inactivation, tubes containing the stock solution were heated at the times and temperatures listed in Table 1. The number of tubes heated at each temperature depended on the dilution required for assay of activity, the minimum number being ten. Two replicates at each time-temperature combination were run. Assay for activity was made before and immediately after heating.

To study the effects of degree of thermal process and storage time on regeneration, the stock solution was heated at the times and temperatures listed in Tables 5–7. For effect of degree of thermal process on regeneration rate, the tubes were assayed for activity at 0, 6, 12, and 20 hr and at 1, 2, 5, 10, 20, and 30 days of storage at 30°C. The experiment was run in duplicate with three replicates per assay for a total of six assays per time-temperature combination.

To study the effects of storage temperature on regeneration rate, the process variable (degree of heating) was standardized. The stock solution was heated 2 min at 135°C; equal lots were stored at 20, 30, and 40°C, and assayed for activity at 0, 1, 2, 5, 10, 20, and 30 days of storage.

RESULTS AND DISCUSSION

Assay procedure. The spectrophotometric trace of absorbance vs. time for the GOR assay was a curve with an approximately linear portion. A line was drawn to the curve in the near-linear region, and the slope of this line was taken as an index of the rate of guaiacol oxidation. Fig. 1 indicates that the velocity of reaction, measured in the manner described above, was proportional to the enzyme concentration. The regression-line equation was $A = 6.79 \times 10^5 C + 1.57 \times 10^{-5}$, where A was absorbance change per second and C was enzyme concentration in moles per liter. The standard error of estimate was zero for the three-place accuracy of the instrumental method.

Inactivation studies. Thermal inactivation of peroxidase heated in the range 85–150°C was shown to follow the typical pattern of first-order reaction kinetics. The rate constants are listed in Table 2, where $k = 2.3/D$. When log k was plotted vs. the reciprocal of absolute temperature (Fig. 2), a linear function was obtained. The

Table 1. Thermal inactivation of peroxidase.

Heating temperature (°C)	Heating time (min)	Corrected time [a] (min)	Active concentration of peroxidase (moles per liter)
85	0	0	500
	8	8	440
	16	16	406
	32	32	276
	64	64	178
	128	128	64.0
	250	250	8.80
100	0	0	510
	4	3.9	406
	7	6.9	356
	10	9.9	311
	20	19.9	164
	60	59.9	14.4
	80	79.9	3.90
115	0	0	440
	1.5	1.43	328
	2.5	2.43	281
	3.5	3.43	241
	7	6.93	96.0
	15	14.93	23.3
	23	22.93	3.94
120	0	0	500
	5	4.9	105
	9	8.9	29.4
	20	19.9	0.980
	30	29.9	0.050
130	0	0	500
	0.6	0.54	341
	1.8	1.74	132
	3.6	3.54	36.1
	7.0	6.94	4.20
	8.5	8.44	0.800
	10.0	9.94	0.280
135	0	0	500
	2.0	1.9	52.5
	3.0	2.9	12.8
	4.0	3.9	3.50
	5.0	4.9	0.956
145	0	0	534
	0.5	0.4	234
	1.0	0.9	67.9
	2.5	1.4	22.5
	2.0	1.9	4.92
	2.5	2.4	1.32
	3.0	2.9	0.480
150	0	0	500
	0.75	0.61	55.9
	1.60	1.46	3.42
	2.20	2.06	0.447
	2.33	2.19	0.283

[a] Corrected time is the thermal energy value of the process, expressed in terms of minutes of holding at constant temperature.

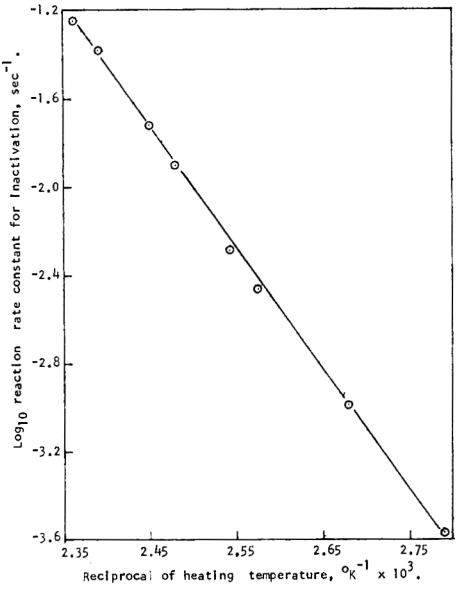

Fig. 2. Effects of temperature on the first-order reaction-rate constant for inactivation of peroxidase.

slope of the regression line, $E/2.3R$, was used to calculate the experimental activation energy, E, 25.1 kilocalories per mole, and since $H = E - RT$, the enthalpy change, ΔH, was determined (Table 3).

The postulates of Eyring (1935, 1938) were assumed to pertain to the inactivation process, namely that the reaction proceeded through an activated state that was rate-limiting, and that the rate constant was an exponential function of the enthalpy and entropy changes, as follows:

$$k = \kappa \left(\frac{RT}{Nh}\right)\left(e^{\frac{-\Delta H}{RT}}\right)\left(e^{\frac{-\Delta S}{R}}\right)$$

where k is the inactivation rate constant, sec^{-1}; N is Avogadro's number; h is Planck's constant; ΔS is entropy change, calories per mole ° K; and κ is a dimensionless transmission coefficient expressing the fraction of activated molecules which become catalytically inactivate.

The above-mentioned relation was used to calculate the entropy changes for thermal inactivation of peroxidase (Table 3). The negative entropy values were particularly significant because large positive entropy terms had been reported previously

Table 2. First-order reaction-rate constants and D values for inactivation of peroxidase.

Heating temperature (°C)	Rate constant, k ($sec^{-1} \times 10^3$)	D value [a] (min)
85	0.269	143
100	1.02	37.7
115	3.46	11.1
120	5.13	7.48
130	12.4	3.11
135	19.2	1.81
145	41.4	0.927
150	56.5	0.678

[a] Slope index of the rate-of-inactivation curve for peroxidase preparation E_2.

for other protein denaturations (Sizer, 1943). On the basis of Stearn's (1949) hypothesis that $N = (H-20)/4 + 1$, where N is the number of hydrogen bonds ruptured and H is in kilocalories, $N = 2$. Since the entropy change for H-bond rupture is roughly 12 e.u., ΔS should have been 24 e.u. instead of -7 e.u. Thus, it seems that the change in the tertiary structure of the protein portion of the enzyme molecule involves more than H-bond and disulfide-bond (no entropy change) rupture. Nevertheless, the over-all picture of the activation stage for thermal inactivation of peroxidase is that the activated molecule is more rigid than the native molecule.

Thermal processing parameters. The kinetic data were used in determining survivor curves for thermal destruction. Table 2 lists the D-values obtained from these curves. These values were used in obtaining the thermal-destruction-time (TDT) curve

Table 3. Thermodynamic constants for inactivation of peroxidase.

Temperature (°C)	Experimental activation energy (kcal per mole)	Enthalpy change (kcal per mole)	Free energy change (kcal per mole)	Entropy change (cal per mole °K)
85	25.1	24.3	27.0	−7.39
100	25.1	24.3	27.1	−7.57
115	25.1	24.3	27.3	−7.84
120	25.1	24.3	27.4	−7.89
130	25.1	24.3	27.4	−7.74
135	25.1	24.2	27.4	−7.68
145	25.1	24.2	27.4	−7.65
150	25.1	24.2	27.5	−7.76

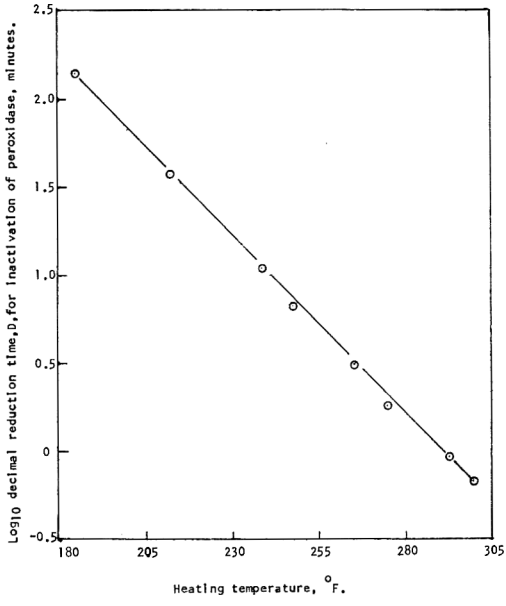

Fig. 3. Thermal-destruction-rate curve for inactivation of peroxidase.

(Fig. 3), from which the z value was found to be 49.8°F.

The z value was within the range previously reported for other plant peroxidase inactivations, although the thermal destruction times were considerably larger than previously reported values for plant peroxidases in natural media (Table 4). The discrepancies were attributed to environmental differences (e.g., pH, ionic strength, cellular materials, heating conditions) and to the assay procedure.

In this study, a pure system was used, and the active concentration of peroxidase was readily assayed without extraction procedures. When the peroxidase system was studied in plant tissues, however, extraction was necessary before assay. The extraction procedures commonly employed (e.g., blending with water or acetone, followed by filtration) probably do not extract all of the active enzyme, leading to erroneous conclusions concerning the true activity of a partially inactivated system, particularly for the case of low residual enzyme activity.

Regeneration after inactivation. Tables 5–8 summarize the results of the regeneration studies. The rates of peroxidase re-

Table 4. z values for the inactivation of peroxidase in low-acid media.

Medium	Highest experimental temperature (°F)	z	Calculated resistance [a] (min) at: 180°	250°	300°	Reference
HRF in $2O_4$ buffer [b]	302	49.8	[c]	[c]	[c]	This study
HRF in FO_4 buffer	302	64.3	314	25.7	4.4	This study
Peas	260	52.0	180	7.7	0.79	Guyer and Holmquist (1954)
Yellow turnips [b]	300	72.5	29.0	3.1	0.64	Esselen, unpubl.
White turnips [b]	300	57.5	35.0	2.1	0.29	Esselen, unpubl.
Broccoli [b]	300	63.0	18.2	1.4	0.24	Esselen, unpubl.
Green beans [b]	300	86.0	3.8	0.59	0.16	Esselen, unpubl.
Peas [b]	300	48.0	170	6.0	0.55	Farkas *et al.* (1956)
Green beans	250	47.0	96	3.0	0.26	Zoueil and Esselen (1959)
Turnips	270	46.0	420	11.3	0.93	Zoueil and Esselen (1959)

[a] Resistance here indicates amount of time required for apparent complete inactivation.
[b] Regeneration occurred.
[c] Where regeneration occurred, inactivation was not complete.

Table 5. Regeneration of peroxidase heated at 120°C.

Storage time (days)	Heating time (min) 5	9	20	30	40
0	104.5 [a]	29.4 [a]	0.98 [a]	0.05 [a]	0
0.25	107	29.7	0.98	0.05	0
0.5	108	29.8	1.02	0.05	0
0.83	111	29.8	1.01	0.05	0
1	181	47.8	3.30	0.27	0
2	168	49.9	2.79	0.24	0
5	139	31.7	2.31	0.14	0
10	147	37.8	2.42	0.16	0
20	154	41.3	2.38	0.17	0
30	141	39.6	2.41	0.17	0

[a] Concentration of peroxidase, moles per liter $\times 10^9$.

generation could not be determined on the basis of reaction kinetics. However, there were several factors of particular importance to the objectives of this study.

It was found that there was a lag period of approximately 20 hr in establishing regeneration. This was attributed to a definite relaxation time of certain bonds critical to the partial renaturation necessary before the protein-porphyrin bonds could be reformed.

Maximum regneration occurred in 2–10 days of storage after inactivation. The longer times (10 days) were required following the higher temperature processes. After maximum regeneration, there was a decrease in activity that was particularly noticeable for samples with the most extensive regeneration. Thus it appeared that the regenerated molecule was less stable than the native molecule.

When peroxidase had been completely inactivated (as evidenced by no activity using the GOR assay) there was no regeneration. Although this finding was contrary to existing reports on plant peroxidases (Guyer and Holmquist, 1954; Zoueil and Esselen, 1959), the authors believe that the results reported here are valid. This discrepancy is attributed to previously mentioned differences in environment and assay.

The effects of storage temperature on regeneration followed the general pattern of the effects of temperature on reaction rates. The higher the storage temperature, the more rapid was the regeneration rate, and the more rapid was the decrease in activity after maximum regeneration.

Table 6. Regeneration of peroxidase heated at 135°C.

Storage time (days)	Heating time (min) 2	3	4	5	7
0	52.5	12.8	3.50	0.956	0
0.25	53.1	12.9	3.71	0.991	0
0.5	52.7	12.8	3.53	0.972	0
0.83	52.6	12.8	3.56	0.988	0
1	54.6	19.5	4.44	2.22	0
2	54.2	19.2	4.36	2.27	0
5	53.7	18.1	4.14	1.93	0
10	52.8	18.7	4.21	2.09	0
20	53.6	18.4	4.17	2.01	0
30	52.7	18.0	4.13	1.87	0

Table 7. Regeneration of peroxidase heated at 150°C.

Storage time (days)	Heating time (min)				
	0.75	1.6	2.2	2.33	3.00
0	55.9	3.42	0.447	0.283	0
0.25	56.2	3.45	0.449	0.294	0
0.5	56.2	3.44	0.451	0.293	0
0.83	56.0	3.42	0.448	0.291	0
1	56.5	3.48	0.468	0.303	0
2	56.8	3.51	0.478	0.319	0
5	57.2	3.56	0.494	0.332	slight +
10	58.6	3.64	0.500	0.351	0
20	56.6	3.46	0.474	0.292	0
30	56.0	3.43	0.449	0.284	0

Table 8. Effects of storage temperature on the regeneration rate of peroxidase heated 2 min at 135°C.

Storage time (days)	Storage temperature (°C)		
	20	30	40
0	40.3[a]	40.3[a]	40.3[a]
1	41.2	51.6	55.9
2	42.4	58.4	56.5
5	55.2	56.8	38.0
10	56.9	53.4	36.7
20	57.2	48.6	35.3
30	55.5	47.5	29.6

[a] Concentration of peroxidase, moles per liter $\times 10^9$.

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Monoterpene Hydrocarbon Composition of Citrus Oils [a]

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SUMMARY

A method for estimating the total monoterpene hydrocarbon content of citrus oils is described. The procedure consists of adding an internal standard (*n*-butylbenzene) to the oils, spotting the mixture on a chromatostrip, eluting the hydrocarbons, and analyzing the eluate by gas chromatography. The total monoterpene hydrocarbon content is calculated from the weights of *n*-butylbenzene and citrus oil used and from the areas under the peaks of the monoterpene hydrocarbons and n-butylbenzene on the chromatogram. The average percent total monoterpene hydrocarbon content and the standard deviation are given for a selected number of citrus oils. Data also show the relative monoterpene hydrocarbon composition of these oils.

Monoterpene hydrocarbons are the major constituents of citrus oils. Although these compounds are present in large quantities, no simple method of estimating the total amount present in an oil has been available. Tedious distillation techniques have been used in the past. More recently, a silicic acid deterpenation method was employed by Kirchner and Miller (1952) to prepare terpeneless essential oils. They reported the terpene hydrocarbon content of several citrus oils and included not only the monoterpenes but also the sesqui-, di-, and triterpenes, as well as the waxes. A direct gas-liquid chromatographic examination of citrus oils, reported by Clark and Bernhard (1960), presents the monoterpene hydrocarbons as a fraction of the volatile constituents, but does not account for nonvolatile constituents such as coumarin compounds (Stanley and Vannier, 1957) or natural waxes.

The method herein described provides an analytical procedure for estimating the total monoterpene hydrocarbon content of citrus oils. The general procedure is similar to that of Stanley *et al.* (1961) for determining the relative monoterpene hydrocarbon composition of citrus oils, except that a known quantity of *n*-butylbenzene is added initially as an internal standard. This compound was selected as the standard because its retention time on gas-liquid chromatograms exceeds that of the known monoterpene hydrocarbons in citrus oils. It is available in high purity and has the same number of carbon atoms in its structure as the monoterpene hydrocarbons. The latter fact makes it suitable for analyses with a flame ionization detector (basically a carbon counter).

To determine if the detector response to *n*-butylbenzene was similar to that for the monoterpene hydrocarbons and to establish optimum conditions for the procedure, various proportions of *n*-butylbenzene were added to samples of *d*-limonene and to lemon oil, and the mixtures were analyzed. To check on recovery by the general procedure, a hydrocarbon mixture of known composition was prepared and analyzed.

GENERAL PROCEDURE

To determine the total monoterpene hydrocarbon content of citrus oils, a weighed quantity of

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Table 1. Detector response (%) to known mixtures of *d*-limonene and *n*-butylbenzene.

Composition by	Mixture 1		Mixture 2		Mixture 3	
	d-Limonene	*n*-Butylbenzene	*d*-Limonene	*n*-Butylbenzene	*d*-Limonene	*n*-Butylbenzene
Weight	65.1	34.9	50.8	49.2	30.2	69.8
Direct GLC [a]	64.3	35.7	50.3	49.7	30.0	70.0
GLC after elution from strip [a]	64.2	35.8	49.9	50.1	29.5	70.5

[a] Calculated from areas under peaks.

n-butylbenzene is added to a previously weighed sample of oil. For lemon oil, the weight of *n*-butylbenzene should be about 85% of the weight of the oil sample; for orange and grapefruit oils, 90%; and for lime oil, 65% (*cf.* last paragraph of experimental section). A 0.04-ml portion of this mixture is applied to a chromatostrip and eluted downward by irrigation with 2% v/v diethyl ether in *n*-heptane, as described by Stanley *et al.* (1961). One ml of eluate is collected in the downward chromatostrip apparatus. A 50- to 80-μl sample of the eluate is then analyzed by gas-liquid chromatography (GLC) on a ¼-in. × 10-ft stainless-steel column packed with 25% w/w diethylene glycol succinate (DEGS) on 42–60-mesh firebrick. The column is operated at 75–80°C with 120 ml per min helium carrier gas flow rate (20 psi inlet pressure). The apparatus is equipped with a 4-element tungsten-filament thermal-conductivity detector.

In the method of Stanley *et al.* (1961) for determining relative concentrations of the monoterpene hydrocarbons in citrus oils, it was found that all the hydrocarbons were eluted with *n*-heptane from the chromatostrip when 2 ml eluate had been collected. By substituting a mixture of 2% diethyl ether in *n*-heptane for the pure *n*-heptane, only 1 ml of eluate need be collected in order to recover both the monoterpene hydrocarbons and the added *n*-butylbenzene. This change, in addition, reduces the time required for deterpenation from 3 to 2 hours.

EXPERIMENTAL

The detector response to *n*-butylbenzene was compared with that of *d*-limonene. Three samples of a mixture of *d*-limonene (the major monoterpene hydrocarbon of citrus oils) and *n*-butylbenzene were prepared. The *d*-limonene was obtained by vacuum distillation of the monoterpene hydrocarbons of orange oil, which in turn had been recovered by column chromatography on silicic acid according to the method of Kirchner and Miller (1952). The *d*-limonene was further purified by preparative scale GLC. Table 1 gives the weights of *d*-limonene and *n*-butylbenzene, the GLC analyses, and the GLC analyses after elution of the three synthetic mixtures from chromatostrips. The detector response to *n*-butylbenzene is sufficiently similar to that of *d*-limonene that no correction is necessary. Stanley *et al.* (1961) reported that the other monoterpene hydrocarbons present in lemon oil have detector responses similar to that of *d*-limonene. After the chromatostrip deterpenation procedure, the GLC analyses show that no changes in the relative concentrations of the *d*-limonene and *n*-butylbenzene result from this treatment.

To determine the effect of the internal standard concentration on the accuracy of the method, varying proportions of *n*-butylbenzene were added to aliquots of a sample of lemon oil, and the resulting mixtures were carried through the general analytical procedure. The results appear in Table 2. No serious effects were observed, even with relatively wide variations in the proportion of added *n*-butylbenzene. However, in order to minimize errors in calculating areas under the peaks traced by the recorder, it is desirable to have the quantity of *n*-butylbenzene approximately equal to the total quantity of the monoterpene hydrocarbons. To check on recovery by the general procedure, a synthetic mixture was prepared containing 0.232 g *d*-limonene, 0.0391 g citral (the major oxygen-containing compound of lemon oil), and 0.244 g *n*-butylbenzene. The *d*-limonene was purified as described previously, and the commercial sample of citral was purified by preparative-scale GLC. The mixture, having a calculated total monoterpene hydrocarbon content of 85.6%, was analyzed by

Table 2. Total monoterpene hydrocarbon content of a lemon oil measured with varying percentages of added *n*-butylbenzene.

n-Butylbenzene (% of mixture)	Total monoterpene hydrocarbon content (% of oil)
22.4	85.1
28.3	84.6
36.7	82.5
54.1	83.4
79.6	80.5
84.4	84.3

the general procedure and found to have a total monoterpene hydrocarbon content of 84.8%.

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms obtained from the monoterpene hydrocarbon analyses of cold-pressed lemon, lime, orange, and grapefruit oils. Peak areas of each monoterpene hydrocarbon and of *n*-butylbenzene are calculated from the integrator sweeps under each peak. Using the known weights of *n*-butylbenzene (W_B) and oil, the peak area of the *n*-butylbenzene (A_B), and the total of the peak areas of the monoterpene hydrocarbons (A_H), the weight of monoterpene hydrocarbon (W_H) in the oil is calculated as follows:

$$\frac{W_H}{W_B} = \frac{A_H}{A_B} \qquad W_H = \frac{A_H}{A_B} \cdot W_B$$

Then, % total monoterpene hydrocarbon content of oil = (W_H/wt of oil) × 100.

The citrus oils used in this study were commercial samples, the lime oils being both of foreign and domestic origin. The "folded" lemon oils were commercial samples concentrated by vacuum distillation to remove some of the monoterpene hydrocarbons. Table 3 shows analyses for the total monoterpene hydrocarbon content of 100 samples of California lemon oil, 6 samples of Arizona lemon oil, 2 samples of Texas Meyer lemon oil, 2 samples of 5-fold lemon oil, 27 samples of California orange oil, 15 samples of Florida orange oil, 4 samples of Texas grapefruit oil, and 5 samples of cold-pressed lime oil. The relative monoterpene hydrocarbon compositions of these same oils are given in Table 4. Each line adds to 100%; the values are averages for the samples shown in Table 3. All values observed have been included; however, when the peak is small there is considerable experimental error, so undue significance should not be attached to values less than 1%.

Table 3. Total monoterpene hydrocarbon content of some citrus oils.

Citrus oil	n	$\bar{X}$	s	C.V.
Lemon, California	100	80.9	3.3	4.1
Lemon, Arizona	6	84.6	4.7	5.6
Lemon, Meyer, Texas	2	89.7	.....	.....
Lemon, 5-fold	2	37.6	.....	.....
Orange, California	27	89.3	2.6	2.9
Orange, Florida	15	90.5	2.5	2.8
Grapefruit, Texas	4	88.4	1.3	1.5
Lime, cold-pressed	5	68.6	3.9	5.7

n = number of samples analyzed.

$\bar{X}$ = average total monoterpene hydrocarbon content, per cent of oil.

s = standard deviation.

C.V. = Coefficient of variation, times 100.

Note that it is possible to determine the concentrations of the individual monoterpene hydrocarbons as a percent of the original oil by using their relative concentrations as determined from the gas chromatograms (*cf.* Table 4) and the total monoterpene hydrocarbon content as determined in the calculation given above (*cf.* Table 3). This technique will be useful in studying regional variations in citrus oil composition. For example, it appears that there are differences in the total monoterpene hydrocarbon content and in the relative hydrocarbon composition of lemon oils obtained from Arizona desert fruit as contrasted with California coastal fruit. Similar differences have been observed in optical rotation (Ikeda *et al.*, 1961) and in citral content (Poore, 1932;

Table 4. Monoterpene hydrocarbon composition of citrus oils.

Citrus oils	% total monoterpene hydrocarbon content	% relative concentrations of individual monoterpene hydrocarbons						
		α-Pinene	β-Pinene	Sabinene	Myrcene	*d*-Limonene	γ-Terpinene	*p*-Cymene
Lemon, California	80.9	1.8	13.0	1.9	1.1	72.2	10.0	Tr.
Lemon, Arizona	84.6	2.0	6.5	1.0	2.1	79.8	8.6	
Lemon, Meyer, Texas	89.7	1.7	3.1	0.6	1.9	82.9	8.8	1.0
Orange, California	89.3	0.3	[a]	0.2	1.4	98.0	0.1	
Orange, Florida	90.5	0.1		0.1	1.3	98.5		
Grapefruit, Texas	88.4	0.2			1.6	97.3	0.9	
Lime, Cold-pressed	68.6	2.5	14.7	2.3	1.0	68.1	10.7	0.7

[a] Blanks indicate 0 as measured on the ¼-inch × 10-foot DEGS column.

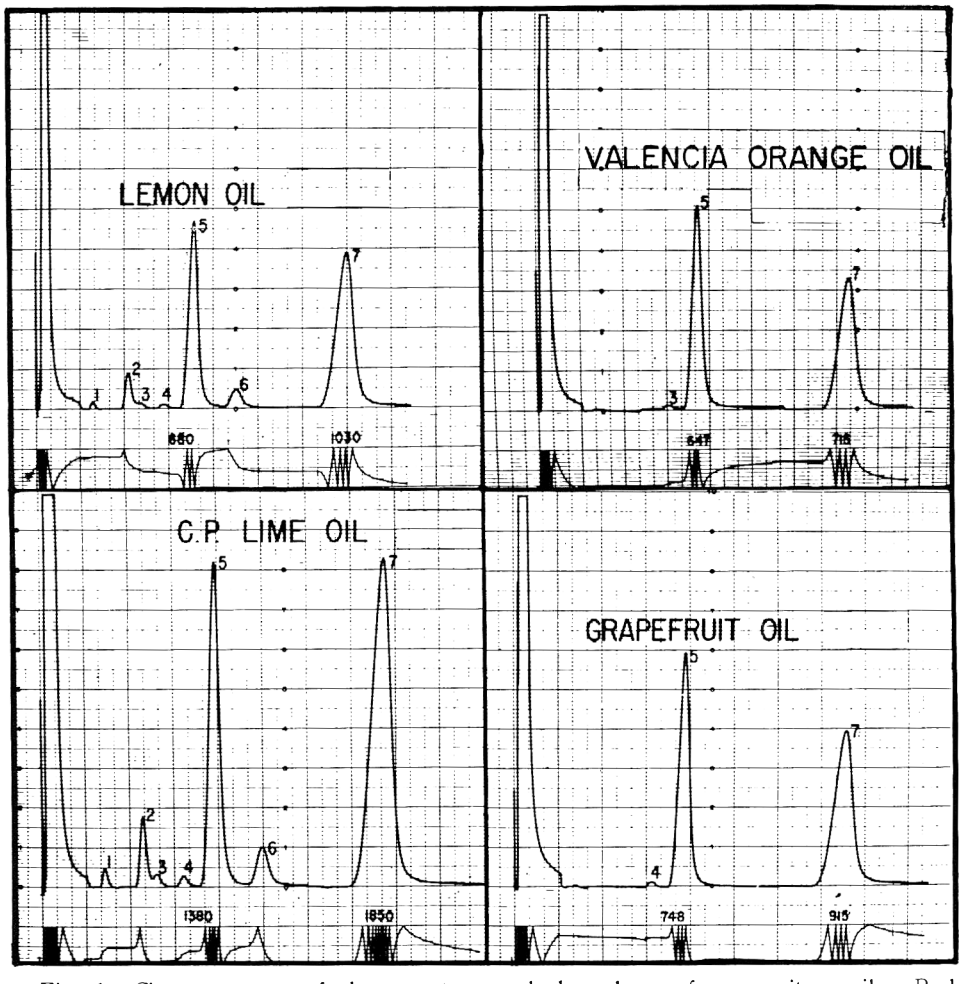

Fig. 1. Chromatograms of the monoterpene hydrocarbons of some citrus oils. Peak identities: (1) α-pinene, (2) β-pinene, (3) sabinene, (4) myrcene, (5) *d*-limonene, (6) γ-terpinene, (7) *n*-butylbenzene. Time: 30 seconds per chart division. Conditions as in text.

Stanley and Vannier, 1959). Known samples of lemon oil made only from coastal fruit generally have total monoterpene hydrocarbon contents of less than 80%, whereas known samples made from desert fruit average about 85%, and in addition, they have only about one-half the β-pinene content of samples made from coastal fruit.

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Preparation and Properties of Rhamnosidase and Glucosidase Fractions from a Fungal Flavonoid Glycosidase Preparation, "Naringinase C-100"

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(Manuscript received August 30, 1962)

SUMMARY

A fungal enzyme preparation, "Naringinase C-100," was separated by paper electrophoresis into individual rhamnosidase and glucosidase fractions. The specificities of these fractions were determined by testing their ability to hydrolyze various flavonoid and related phenolic glycosides. The individual rhamnosidase and glucosidase fractions were characterized by determining the degree of hydrolysis produced in standard naringin and prunin substrate solutions at various pH's, temperatures, and time intervals. A paper chromatographic-fluorometric method suitable for quantitative determination of naringin and prunin in mixtures containing naringin, prunin, and naringenin was devised and employed in the characterization studies.

INTRODUCTION

Thomas and co-workers (Thomas *et al.*, 1958) recently reported on the enzymatic action of their partially purified fungal enzyme preparation, "Naringinase C," on naringin (4′,5,7-trihydroxyflavanone-7-rhamnoglucoside), the main bitter principle in grapefruit. Their "naringinase C" was shown indirectly to contain a rhamnosidase and a glucosidase capable of converting naringin to the aglycone naringenin (4′,5,7-trihydroxyflavanone) via the intermediate glucoside prunin (naringenin-7-glucoside), e.g.,

$$\text{naringin} \xrightarrow{\text{rhamnosidase}} \text{prunin} \xrightarrow{\text{glucosidase}} \text{naringenin}$$

The rate of over-all conversion of naringin to naringenin in aqueous citrate buffer in their studies was followed by the Davis method (Davis, 1947). This method is based on the yellow color produced by naringin but not by naringenin, upon reacting with alkaline-diethylene glycol, as measured spectrophotometrically at 420 mμ.

Thomas *et al.* (1958) found that "naringinase C" rapidly debittered natural grapefruit juices, but they could not correlate debittering with the decrease in naringin content obtained by analysis with the Davis test. This was found to be the result of the accumulation in juice of the non-bitter intermediate prunin, which occurred because of inhibition of the glucosidase in the enzyme preparation by glucose present in the juice. Since prunin gives essentially the same color reaction as naringin with the Davis reagent, the Davis method was found useless for measurement of naringin in the presence of prunin.

In the study reported here, the actual separation of a "naringinase C" preparation into individual glucosidase and rhamnosidase fractions was achieved, and the specificity of these fractions in hydrolyzing a number of flavonoid and phenolic glycosides was determined.

A paper chromatographic-fluorometric method suitable for quantitative determinations of naringin and prunin in mixtures containing very small quantities of naringin, prunin, and naringenin was developed. This method was employed to characterize the

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effect of pH and temperature on rates of hydrolysis of naringin and prunin by the rhamnosidase and glucosidase, respectively.

EXPERIMENTAL METHODS

Preparation of glucosidase and rhamnosidase. A Karler-Misco continuous-flow paper electrochromatography cell (Microchemical Specialties Co., Berkeley, Calif.) with a gravity-flow wick sample feeder was used for the preparation of glucosidase and rhamnosidase. A Whatman 3MM chromatography paper curtain was placed in the cell with the table at the intermediate level and equilibrated for several hours with $5.75 \times 10^{-2} M$ borate buffer of pH 8.5. The feeder containing 50 mg of "naringinase C-100" concentrate (comparable to the "naringinase C" described by Thomas *et al.*, 1958; obtained for research work from Rohm and Haas Co.) in 20 ml of the borate buffer was placed in the right-hand window feeder position above the anode buffer chamber. A current of 1.55 ma (318 V) was supplied. The electrophoretic separation was continued for 5 days at about 22°C; approximately one-half of the feed was consumed.

Determination of activity. The resulting 22 electrophoretic fractions were examined for activity by incubating each with standard naringin and prunin solutions. The reaction products were determined by paper chromatography. The standard aqueous substrate solutions consisted of 2.0×10^{-3}% chromatographically pure naringin or prunin, 1.0% citric acid, and sufficient sodium hydroxide to give a pH of 4.0. To individual 10-ml portions of these standard solutions were added 0.2-ml portions from each of the collected electrophoretic fractions. These solutions, together with controls consisting of standard substrate only, were incubated 6 hr at 50°C, and the incubated solutions were immediately extracted with redistilled ethyl acetate.

The extracts were evaporated to dryness and taken up in 1-ml portions of redistilled methyl alcohol. Portions of 100 λ each were chromatographed, together with reference samples of naringin, prunin, and naringenin, on Whatman no. 1 paper in the *n*-butyl alcohol–acetic acid–water (BAW) solvent system (6:1:2,v/v/v). The resulting chromatograms were sprayed with 1% aluminum chloride in methyl alcohol and examined under ultraviolet light (3660Å, Blak-Ray, Ultraviolet Prod., Inc., San Gabriel, Calif.) to locate fluorescent compound zones.

In this manner, electrophoretic fractions 2–5 (numbered from the cathode buffer chamber) were found to hydrolyze prunin to naringenin, but they did not alter naringin. These electrophoretic fractions were combined as "glucosidase." Fractions 14–20 hydrolyzed naringin to prunin, but did not affect prunin. These were combined as "rhamnosidase." Other fractions either contained no active material or contained a mixture of glucosidase and rhamnosidase, and were discarded. In no case was hydrolysis found in the controls, which contained only standard substrate solutions.

Specificity studies. The specificity of the rhamnosidase and glucosidase fractions was checked by determining their activity on a number of other available flavonoid and phenolic glycosides. Solutions of these glycosides in citrate buffer of pH 4.0 were incubated at 50°C with portions of the

Table 1. Effect of rhamnosidase and glucosidase on certain glycosides.

Glycoside tested	Sugar moiety hydrolyzed	
	Glucosidase	Rhamnosidase
Quercetin-3-glucoside (isoquercitrin)	glucose	none
Naringenin-7-glucoside (prunin)	glucose	none
Esculetin-6-glucoside (esculin)	glucose	none
Scopoletin-7-glucoside (scopolin)	glucose	none
Hesperetin-7-glucoside	glucose	not tested
Apigenin-7-glucoside	glucose	not tested
Isosakuranetin-7-glucoside	glucose	not tested
Quercetin-3-rhamnoglucoside (rutin)	none	rhamnose
Quercetin-3-rhamnoside (quercitrin)	none	rhamnose (slightly)
Naringenin-7-rhamnoglucoside (naringin)	none	rhamnose
Kaempferol-7-rhamnoglucoside	not tested	rhamnose
Apigenin-7-rhamnoglucoside (rhoifolin)	not tested	rhamnose
Hesperetin-7-rhamnoglucoside (hesperidin)	not tested	rhamnose
Neohesperidin	not tested	rhamnose
Isosakuranetin-7-rhamnoglucoside	not tested	rhamnose

enzyme preparations, and the reaction products were identified by paper chromatography as described above.

Quantitative measurement of naringin or prunin. Naringin and prunin standard substrates, as described above, were used respectively in studying rhamnosidase and glucosidase activity.

Aliquots of enzyme solution were added to 10-ml portions of standard substrate solution of the indicated pH. The resulting solutions were incubated at the desired temperature for the required time. Immediately upon removal from the incubator, the reaction mixtures were extracted with four 15-ml portions of redistilled ethyl acetate. Practically quantitative extraction of prunin and naringin was obtained by this method.

The extracts were evaporated to dryness and taken up in 1 ml redistilled methyl alcohol, of which 100 λ was spotted on Whatman No. 1 paper. Papers were developed in BAW for 14 hr to separate naringin from prunin in the rhamnosidase studies, and in distilled water for 5 hr to separate prunin and naringenin in the glucosidase studies. The papers were dried thoroughly at room temperature, then sprayed with 1% aluminum chloride in methyl alcohol solution. The sprayed papers were allowed to develop 24 hr at room temperature. Spots of either naringin or prunin were cut from the papers after marking equal areas for each spot viewed under uv light. Each spot was cut into small pieces with scissors and placed in a 25-ml stoppered flask containing 10 ml of 1% methanolic aluminum chloride. The flasks were shaken 20 min, and the contents then allowed to settle. Portions from each sample were decanted into cuvettes, and the intensity of fluorescence of the prunin-$AlCl_3$ complex or naringin-$AlCl_3$ complex at 520 mμ, produced by an activating wavelength of 325 mμ, was measured in an Aminco-Bowman spectrophotofluorometer. The quantity of prunin or naringin in the sample was then determined from a standard curve obtained by carrying 2.5–20-μg quantities of appropriate standard glycoside through analogous chromatography and elution steps, and plotting percent transmission (intensity) vs. concentration of glycoside.

RESULTS AND DISCUSSION

Specificity studies. The results of the specificity studies are compiled in Table 1. It can be seen that the glucosidase specifically hydrolyzed glucosides, and that no hydrolysis occurred at rhamnoglucoside-aglycone linkages. Other limitations to glucosidase action are not readily apparent; however, the mode of glucoside attachment is generally thought to be beta for the flavonoid glucosides used in this study. The rhamnosidase specifically hydrolyzed the rhamnosides tested in which the rhamnose was attached to glucose, as in rhamnoglucosides, and also hydrolyzed the one rhamnoside tested in which the rhamnose was attached directly to the aglycone (quercetin-3-rhamnoside), although less readily than in the case of the rhamnoglucosides.

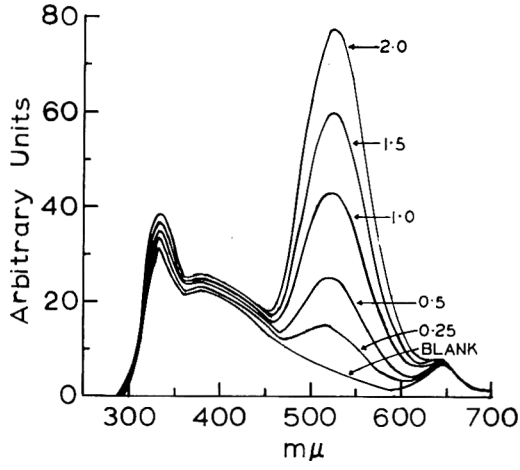

Fig. 1. Typical fluorescent spectra for naringin or prunin in 1% $AlCl_3$ in methyl alcohol produced by an activating wavelength of 325 mμ with the fluorescence maxima at 520 mμ. Concentration range studied was 0.25 to 2.0 μg/ml.

Quantitative measurement of naringenin or prunin. Fig. 1 shows typical fluorescence spectra for various concentrations of prunin or naringin in 1% aluminum chloride in methyl alcohol solution. Within

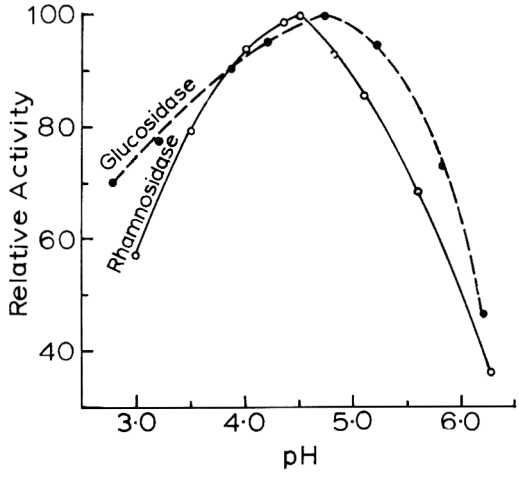

Fig. 2. Effect of pH on activity.

the concentration range 0.2–2 μg/ml, percent transmission was found to be a linear function of naringenin-glycoside concentration; above this concentration range, fluorescence quenching apparently became significant, and percent transmission dropped off.

Repeated experiments revealed that, although elution of prunin or naringin from the paper was not complete under the conditions employed, for each individual paper the proportion eluted of compound actually applied to the paper was essentially constant for all samples within the concentration range of 2.5–20 μg; hence, the linearity of the percent transmission-concentration relationship was maintained. However, the proportion eluted of samples of the same size applied to different papers often varied

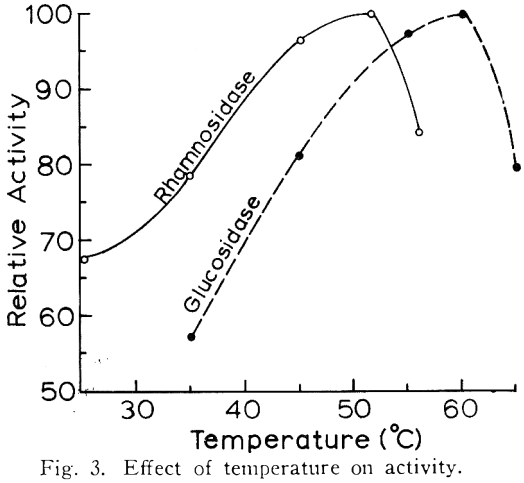

Fig. 3. Effect of temperature on activity.

slightly, thus requiring spotting of three or four standards for construction of standard curves for each paper run if highest accuracy was to be obtained. Determination of naringenin by this procedure was not employed in this work, since the solvent system, distilled water, used to separate prunin and naringenin produced too much streaking of naringenin spots on chromatograms.

Rate of hydrolysis studies. Figs. 2 and 3 show the effect of pH and temperature on rates of hydrolysis, using the developed quantitative method for measuring prunin. For rhamnosidase the optimum pH region is 4.5 when incubated 2.5 hr at the optimum temperature of 50°C. The optimum temperature for glucosidase at optimum pH 4.75 is 60°C when incubated 3 hr. Using the above optimum conditions, the rates of hydrolysis were determined. Fig. 4 shows hydrolysis of standard naringin solution by rhamnosidase (0.05 ml enzyme/ml substrate solution) as evaluated, measuring prunin. It can be seen that the curve is linear to 70% hydrolysis, thus indicating zero-order kinetics in the early stages of reaction. First-order kinetics are indicated with glucosidase. Fig. 5 shows a plot of $\log a/(a-x)$ where a is the initial prunin concentration and x the concentration of prunin converted by glucosidase (0.04 ml enzyme/ml substrate solution).

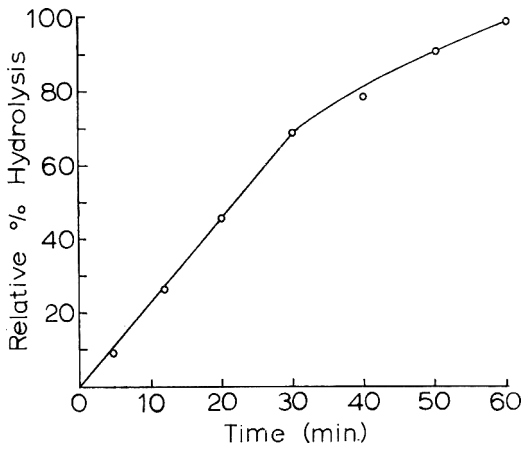

Fig. 4. Hydrolysis of standard naringin solution by rhamnosidase.

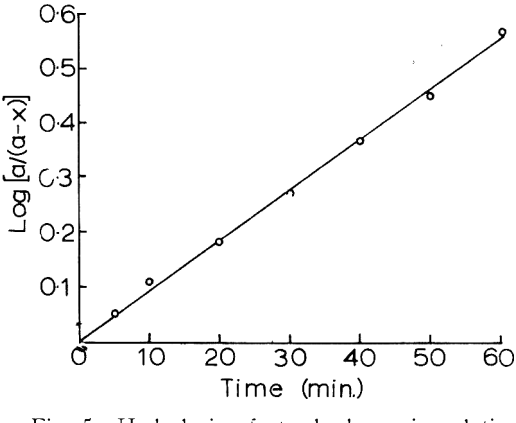

Fig. 5. Hydrolysis of standard prunin solution by glucosidase where a = initial prunin concentration and x = concentration of prunin hydrolyzed.

ACKNOWLEDGMENTS

The authors thank Dudley W. Thomas, Rohm and Haas Co. Research Laboratories, Bristol, Pa., for kindly supplying the "Naringinase C-100" concentrate used.

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Pesticide Residue Analysis. Gas Chromatographic Analysis of 2,6-Dichloro-4-nitroaniline

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(Manuscript received August 24, 1962)

SUMMARY

Two procedures for the analysis of dicloran (2,6-dichloro-4-nitroaniline) by gas chromatography are described. Determination of residues in fruit with a microcoulometer detector with a sensitivity of 0.01 ppm is outlined, and determination of dicloran with a thermal-conductivity detector with a sensitivity of 0.5 ppm is discussed. Gas chromatography with a thermal-conductivity detector is also applicable to the analysis of formulations of dicloran for quality control or regulatory purposes.

Postharvest fungus infestation on stone fruits has been shown by Ogawa *et al.* (1961) to be controlled by 2,6-dichloro-4-nitroaniline (dicloran). Fruits treated after harvest but before storage for ripening and processing showed effective control of these fungi. Recent information indicates that a preharvest spray treatment of fruits also controls postharvest fungus rots. In this case also, a sensitive and accurate method for determining the residue levels is important.

Anticipated greater use of 2,6-dichloro-4-nitroaniline as a fungicide for fruits presents the need for sensitive methods of analysis. The availability of alternate methods is often important to pesticide residue chemists.

A previous paper from this laboratory (Kilgore *et al.*, 1962) described a colorimetric method of analysis for the determination of 2,6-dichloro-4-nitroaniline (dicloran) in processed fruits. The method included an extraction and cleanup procedure for several varieties of canned peaches and apricots. The analysis of both fruit and syrup was discussed.

As an extension of that work, and using the Kilgore methods of extraction and cleanup, two gas chromatographic methods of analysis were tested and found to be effective. One of these methods uses a thermal-conductivity detector, and the other a microcoulometer detector. The thermal-conductivity detector will respond to as little as 5 μg, and the microcoulometer will respond to the chlorine from as little as 0.3 μg of dicloran.

EXPERIMENTAL

An experimental run on dicloran with the thermal-conductivity detector gave a single well-resolved peak (Fig. 1) at 205°C at a programmed rate of temperature increase of 11°/min. Confirmatory runs programmed at 21°/min also showed a sharp peak at 250°C (Table 1). This temperature change was expected since the rate of temperature rise was increased. With isothermal operation at 180°C a response may be obtained in a reasonable time, though at a sacrifice of peak height and shape.

A similar standard solution was injected into the gas chromatograph with the microcoulometer detector at 210°C. A response was obtained in 4 min, showing that this detector was well suited for use in the analysis of dicloran (Fig. 2).

Table 1. Response of thermal-conductivity detector to standard solutions of dicloran.

μg added	Volume (μl)	Av. peak height (mm)	Av. variation (mm)
40	50	204	8
20	25	95	0
10	12.5	49	2
5	6.25	22	2
1	1.25	6	1

Instrument equipped with a 2-ft stainless-steel column, ¼-in. O.D., packed with 20% fractionated Dow-11 silicone grease on 40–50-mesh Chromosorb P, programmed at 21°C/min with a gas flow rate of 50 ml He/min, bridge power 200 m.a. Injection port and detector block, 275°C.

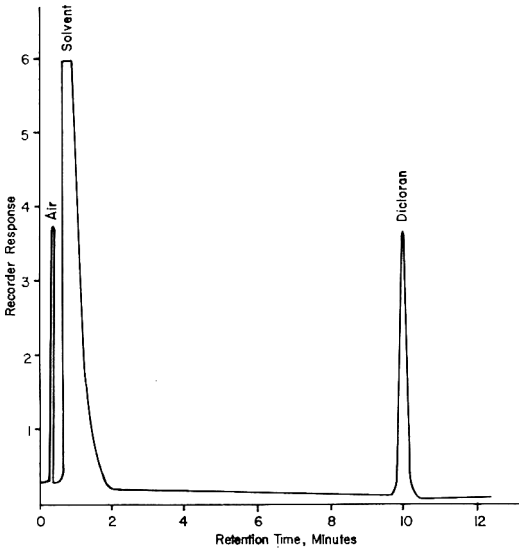

Fig. 1. Gas chromatographic response curve for dichloran using thermal-conductivity detector and program temperature. Operating conditions: Initial temp. 70°C, program 21°/min, He flow rate 55 cc/min, attenuation 1×, bridge power 200 m.a., block 275°, 20 μg dicloran, response at 225°C, stainless-steel column ¼ in., 2 ft long, packed with 30/60-mesh Chromosorb P coated with 20% (w/w) Dow-11 silicone oil.

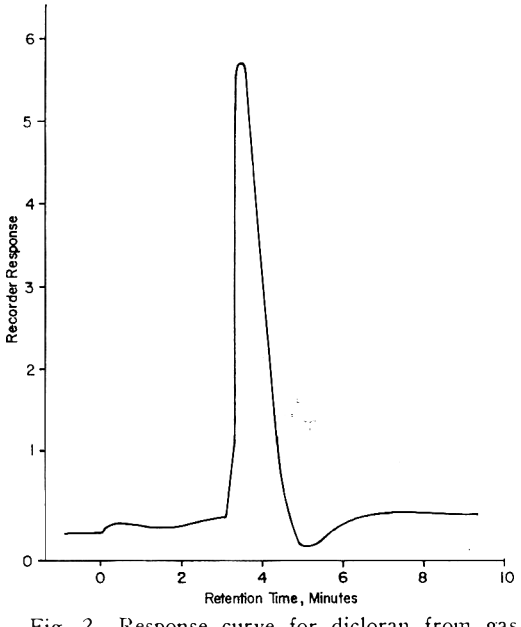

Fig. 2. Response curve for dicloran from gas chromatograph using microcoulometer detector. Operating conditions: Block temp. 245°, column temp. 210°, sensitivity 64 ohms, 5 μg dicloran, carrier gas (N_2) flow rate 100 ml/min, oxygen flow rate 100 ml/min, quartz column 6-ft, 5 mm O.D., packed with 20% fractionated Dow-11 silicone grease on 40/50-mesh acid-washed Chromosorb P.

Table 2. Recovery of dicloran as a measure of column and instrument efficiency using the microcoulometer detector.

μg added	Detector resistance (ohms)	% recovery average	
		aluminum [a]	quartz [b]
5.0	64	82.4	97.0
4.0	64	83.8	97.0
3.0	128	83.3	92.6
2.0	64	82.0	95.5
1.5	128	79.2	.....
1.0	64	80.0	94.8
0.5	512	75.6	.....
0.3	512	70.0	.....

[a] Instrument equipped with a 6-ft aluminum column, ¼-in O.D., packed with 20% fractionated Dow-11 silicone grease on 40/50-mesh acid-washed Chromosorb P. Injection block, 245°C, column, 212°C.

[b] Quartz column, 5 mm diam, in place of aluminum.

The extraction and cleanup procedures described by Kilgore *et al.* (1962) were tested and found to remove all extractives from canned peaches and apricots that would cause any interference in the gas chromatographic analysis of dicloran. It was also determined that the extracts of syrup need only to be concentrated for gas chromatographic analysis, thus eliminating the need for the Florisil cleanup step.

METHOD

Fruit or syrup is prepared for analysis by extraction as described by Kilgore *et al.* (1962). A 100-ml portion of a sample representing 50 g of fruit or 25 g of syrup is put on a 2.5 × 25-cm column containing 25 g of Florisil previously rinsed with 50 ml of benzene. The Florisil is used as purchased. All of the eluate is retained, along with the 200 ml of 1% ethyl ether in benzene used as wash solution.

Extracts of syrup or Florisil eluates are evaporated under reduced pressure with a rotating flash evaporator just to the point of dryness. The flask is removed from the evaporator, and the inner walls are washed down with a small volume of ethyl ether. The ether solution is transferred quantitatively to a graduated sedimentation tube for further concentration. When the volume reaches 100–300 μl, the exact amount is recorded and an aliquot is taken for injection into the gas chromatograph.

The gas chromatograph with the microcoulometer detector was used for residue analysis. A sensitivity of 0.05 ppm is readily obtained. The sensitivity has been pushed to a lower value, and is shown in Table 3. The thermal-conductivity detector could readily give a sensitivity of 0.5

Table 3. Residues and recovery of dicloran from treated apricot fruit and syrup.[a]

Sample	Dicloran residue (ppm)[b]		% Recovery of fortified check[c]	
	Fruit	Syrup	Fruit	Syrup
Check 1	0.0	0.0	80	75
Treatment 1	0.04	<0.01	....	....
Check 2	0.0	0.0	85	80
Treatment 2	0.02	<0.01	....	....

[a] Fruit and syrup are the same as the 1000- and 750-ppm treatments described by Kilgore *et al.* (1962).

[b] Sensitivity set at 0.01 ppm using the instrument with the microcoulometer detector and conditions as given in Table 2.

[c] Check samples fortified at the 1-ppm level.

ppm, which would be sufficient for survey work.

Formulation analysis may be easily and rapidly done using a chromatograph with a thermal-conductivity detector. By dissolving an aliquot of the formulated product (Botran, Upjohn Company) in acetone (3.4% solubility), a response is obtained that may be used for quantitative measurement. A 1% solution is equivalent to 10 μg/μl, which is a suitable strength for analysis.

MATERIALS AND EQUIPMENT

Gas chromatographs:

1) F and M Model 500 with thermal conductivity detector.
2) Dohrmann Model G-100 with standard microcoulometer.

Florisil:

60/100-mesh, the Floridin Co., Tallahassee, Fla.

RESULTS AND DISCUSSION

The linearity of response of the thermal-conductivity detector was measured over the range of 1–40 μg with varying volumes of injection. The results and column operating conditions are shown in Table 1. The peak height shows the linearity of response, and the average variation in peak heights shows the reproducibility of the instrument and injection procedure.

The percentage recovery of dicloran standards using the microcoulometer detector is shown in Table 2. A comparison of recovery between an aluminum and a quartz chromatographic column is shown at various levels of instrument sensitivity. The conditions of column operation are described. Improved recovery using the quartz column has also been observed with several other pesticidal compounds. The microcoulometer measures the halogen content of the sample, and the results are based on comparison with the theoretical chlorine content.

Table 3 presents the results of analysis of apricot fruit and syrup samples and the recovery of laboratory fortified samples carried through the entire analytical procedure. The gas chromatograph with the microcoulometer detector was used to obtain the data shown. The residue values shown are in agreement with the data obtained by Kilgore *et al.* (1962) on the same samples. Other confirmatory data were obtained in the method development, using portions of the samples prepared for analysis by Kilgore *et al.* in development of their method.

Fig. 1 shows the response curve for dicloran using the thermal-conductivity detector. The response is sharp, indicating that the compound is essentially pure, without any related compounds or isomers being present.

Results were similar with the microcoulometer detector, but with a broader peak (Fig. 2). This is characteristic of this type of detector, and the peak shape is a result of the slow response of the detector.

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Ogawa, J. M., S. D. Lyda, and D. J. Weber. 1961. Postharvest treatment of fruit with 2,6-dichloro-4-nitroaniline for the control of fungus rots. *Plant Disease Reptr.* **24**(8), 636.

The Bioassay of Pimaricin and Its Binding Effect in Orange Juice[a]

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(Manuscript received June 25, 1962)

SUMMARY

The stability of low levels of the antifungal pimaricin in orange juice can be quantitatively followed by a cylinder-plate agar assay using a strain of *Saccharomyces cerevisiae* that is sensitive to 0.6 μg/ml. Pimaricin is stable in orange juice stored in the dark under refrigeration. The assay data indicate that pimaricin is adsorbed or bound to the juice pulp, and the recovery from the juice depends upon proper sample dilution.

INTRODUCTION

This report presents an assay procedure for pimaricin in orange juice and indicates that pimaricin is bound to the pulp fraction of the juice. (The trademark of the American Cyanamid Company for pimaricin is Myprozine.)

The addition of 20 μg/ml and lower concentrations of the tetraene antifungal pimaricin to orange juice has been shown by Shirk and Clark (1962) to control yeast in fresh orange juice. An assay to determine the recovery and stability of pimaricin in orange juice was developed during the present studies.

MATERIALS AND METHODS

Materials. The materials used were:

1) Glass Petri plates with covers, 100 × 22 mm, flat bottom for assay
2) Stainless-steel cylinders, 8 mm outside diameter, 6 mm inside diameter, and 10 mm in length
3) Bacto-peptone, Difco Laboratories
4) Ascorbic acid, U.S.P.
5) Nystatin assay agar, Baltimore Biological Laboratory
6) Glacial acetic acid, reagent grade
7) Sodium hydroxide, reagent grade
8) Dextrose, reagent grade
9) Pimaricin, crystalline material of 97–100% purity
10) Distilled water

Test organism. The test organism, *Saccharomyces cerevisiae* ATCC 9763, was maintained on nystatin assay agar (pH 6.1) slants under normal refrigeration. The organism was transferred about every three months to fresh slants, incubated at 37°C for at least 24 hr, and then refrigerated for storage.

The inoculum of *S. cerevisiae* for the assay was prepared by transferring a loopful of growth from an agar slant culture into 100 ml (250-ml Erlenmeyer flasks) of sterile 1% peptone plus 2% dextrose broth (pH 5.6–5.7), and incubated 18–20 hr at 37°C.

Standard solutions. *Diluent.* A 3% peptone plus 1% ascorbic acid solution adjusted to pH 4.5 was prepared as a diluent for pimaricin and the samples to be assayed. Several flasks, each containing 3% peptone, were prepared by dissolving 10.5 g of peptone in 350 ml of distilled water and sterilizing. The sterile 3% peptone was stored at room temperature and could be used over a period of several weeks without any effect on assay results.

Immediately before use, 3.5 g of ascorbic acid (1%) were added to each 3% peptone flask; the number of flasks to be used was determined by the number of samples to be assayed. The pooled 3% peptone plus 1% ascorbic acid solution was adjusted to pH 4.5 with 3*N* sodium hydroxide, thus providing the diluent for all subsequent standard pimaricin solutions and samples for assay.

Pimaricin standard. The pimaricin standard was prepared in a 100-ml beaker by dissolving 10.0 mg of pimaricin with 2 ml of glacial acetic

[a] Presented at the 22nd Annual Meeting of the Institute of Food Technologists, June 12, 1962, Miami Beach, Florida.

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acid. A clear solution was obtained after about ten seconds of gentle agitation. Immediately after dissolving, the 2 ml of acetic acid-pimaricin solution was brought to 40 ml with diluent and the pH was adjusted to 4.5 with 3*N* sodium hydroxide. The final volume was brought to 50 ml with additional diluent to provide a standard solution of 200 μg/ml of pimaricin.

Standard-curve dilutions. The following solutions were prepared by dilution in 125-ml Erlenmeyer flasks for obtaining the standard curve:

Flask no.	Diluent + pimaricin solution	Final pimaricin standard-curve solutions
		μg/ml
1	42 ml + 8 ml of the 200 μg/ml pimaricin standard	32
2	25 ml + 25 ml of flask No. 1	16
3	25 ml + 25 ml of flask No. 2	8
4	25 ml + 25 ml of flask No. 3	4
5	25 ml + 25 ml of flask No. 4	2
6	25 ml + 25 ml of flask No. 5	1
7	25 ml + 25 ml of flask No. 6	0.5

Assay procedure. A flask of sterile 1% peptone plus 2% dextrose broth was inoculated with the test organism 18–20 hr before preparation of the agar assay plates. At the same time a sufficient number of sterile assay Petri plates were placed in a 55°C oven for overnight storage.

On the following day, flask(s) of nystatin assay agar (300 ml) were melted and placed in a 48–49°C water bath. The pimaricin (10.0 mg) was weighed out and placed in a 100-ml beaker to be set aside until later. The 3% peptone plus 1% ascorbic acid diluent at pH 4.5 was prepared in an amount judged sufficient for the day's work, and added in the designated amounts to the 125-ml Erlenmeyer flasks used for the standard-curve solutions.

When the melted agar was cooled to 48–49°C, it was seeded with 7.5 ml (2.5%) of an 18–20-hr culture of *S. cerevisiae*. Six milliliters of the inoculated agar was added to each plate (preheated to 55°C) with an automatic syringe and evenly distributed by gentle rotation. All poured plates were set aside on a level stone bench to cool and harden. The lid of each plate was canted to the side during cooling to lessen condensation on the underside of the lids. Preheating the assay plates to 55°C ensured a slower cooling of the agar, providing a more uniform set of poured assay plates.

While the plates were cooling, the 200 μg/ml pimaricin standard was prepared as previously described and the standard solutions completed by diluting the pimaricin standard. Six sterile stainless-steel cylinders were placed symmetrically at 60-degree intervals on each agar plate by dropping the cylinders through a specially drilled template. Three plates were used to obtain a standard curve. The cylinders on each plate received, in clockwise order, by pipette, 4–6 drops of the standard solutions of pimaricin (16, 0.5, 8, 4, 2, and 1 μg/ml).

The orange juice samples containing pimaricin were appropriately diluted in standard diluent and pipetted dropwise into the cylinders in triplicate, one cylinder of each sample per plate with six samples per plate. Thus, if 24 prepared orange juice samples were to be assayed in triplicate, it would require 12 plates for the samples and 3 plates for the standard curve. The lids were carefully replaced on the plates after the cylinders were filled. Since pimaricin is labile to light, brown paper was placed over the entire set of plates, which were maintained in their original position, and incubated at room temperature.

After 18–24 hr of incubation at room temperature, the cylinders and test solutions were removed from the plates, and the zones of *S. cerevisiae* inhibition were measured on a Fischer-Lilly antibiotic zone reader. The triplicate zones of inhibition for each assayed sample were averaged. The averaged zones of inhibition of the standard solutions were plotted against concentration on three-cycle semi-log paper and a straight line fitted by eye through the points to obtain the standard curve.

The averaged zones of inhibition for each orange juice sample were compared with the standard curve to obtain the concentration of pimaricin in the sample. The result was multiplied by the dilution factor of the sample to arrive at the concentration of pimaricin in the orange juice.

RESULTS AND DISCUSSION

A typical standard curve for pimaricin is shown in Fig. 1. The 0.5-μg/ml standard of

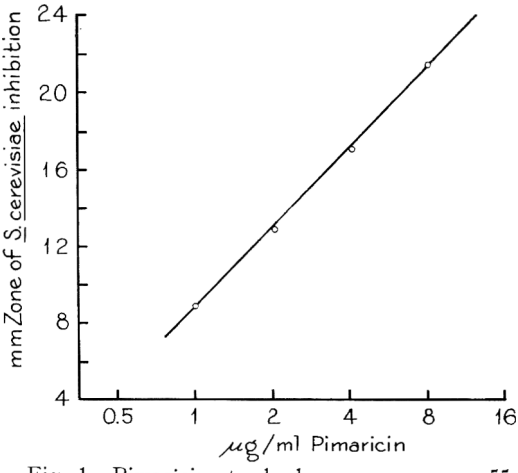

Fig. 1. Pimaricin standard curve, assay no. 55.

pimaricin did not consistently give a response since this concentration approaches the limit of the test organism's sensitivity to pimaricin. Usually, only the values for the 1-, 2-, 4-, and 8-μg/ml standards were plotted. In general the points fell very nearly on a straight line and the calculation and plotting of a regression equation gave no significant advantage over the line fitted by eye.

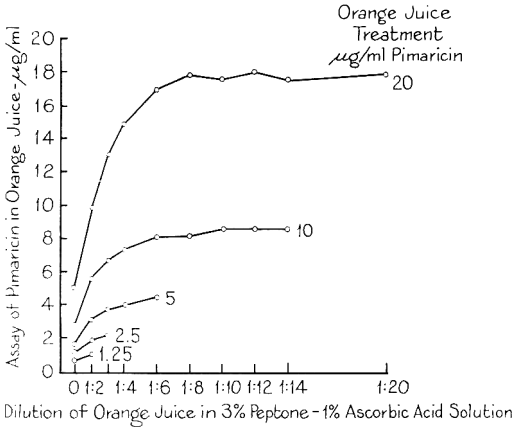

Fig. 2. The effect of sample dilution upon the assay of pimaricin in orange juice treatments.

Preliminary assay results on orange juice containing 20 μg/ml of pimaricin indicated that when the juice was diluted with the standard diluent from 0 to about 1:10 there was a progressive increase in the recovery of pimaricin. The undiluted juice showed about 30% recovery whereas the diluted juice gave progressively increasing values until at about a 1:10 dilution approximately 90–95% of the pimaricin could be accounted for. Fig. 2 shows this recovery effect in orange juice containing pimaricin at 1.25, 2.5, 5, 10, and 20 μg/ml. At all of the pimaricin concentrations the recovery increased with dilution. Thus, accurate assay of pimaricin in the juice required that assay be made at several dilution points to offset the binding action of the juice and that the recovery value be based on the average of the points giving the greatest response. No inhibitory response was obtained with blank samples of whole and diluted juice.

Fig. 3 shows the semi-log graph of the assay results for each concentration of pimaricin in orange juice stored 12 weeks at 2.5°C in the dark. The figure indicates the prolonged stability of pimaricin in orange juice and that the slow rate of pimaricin loss is similar at both high and low concentrations of pimaricin. After 12 weeks of storage, recovery of the pimaricin was respectively 77, 74, 70, 68, and 48% for the pimaricin treatments of 20, 10, 5, 2.5, and 1.25 μg/ml. The fact that the lowest levels of pimaricin gave the lowest percentage of recovery was due to the "binding effect" combined with the sensitivity of the assay, i.e., it is impossible to dilute a low concentration of pimaricin, e.g., 1.25 μg/ml, more than 1:2 and remain within the minimum sensitivity limits of the assay (about 0.6 μg/ml).

It was speculated that this effect was due to the low pH of less diluted orange juice samples. The orange juice has a pH of 3.7, and consequently, when the undiluted juice is placed in the cylinders upon the assay agar, the pH of the sample is 3.7, compared with the standard-curve diluent of pH 4.5. As the juice is diluted the pH rises until, at a 1:8 dilution, it becomes constant at 4.5. Therefore, both the normal orange juice containing pimaricin and the same juice adjusted to pH 4.5 were assayed to test for a pH effect upon assay. None was found.

For further study of the factor in orange juice responsible for increased activity of diluted juice, pimaricin-treated orange juice

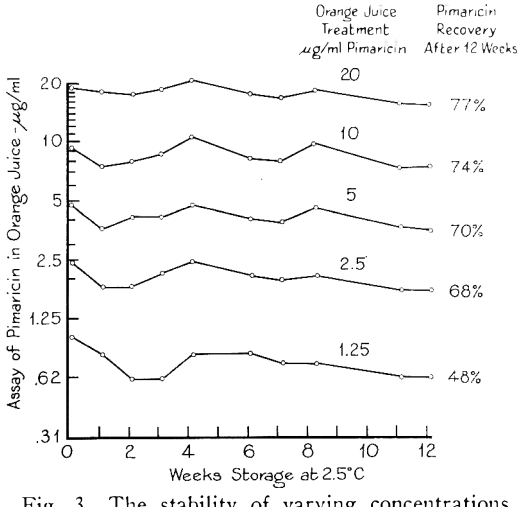

Fig. 3. The stability of varying concentrations of pimaricin in orange juice stored at 2.5°C in the dark.

was separated into its pulp and serum fractions with a clinical centrifuge, and each fraction was assayed separately. Fig. 4 shows the average results of two assays calculated as percent recovery of pimaricin obtained on orange juice containing pimaricin at 20 μg/ml. This figure illustrates the assay results of the whole juice, showing increased pimaricin recovery with increased dilution of the juice. The same juice samples (43.5 ml) were centrifuged, and the serum (38.2 ml) decanted off and assayed in the same manner as the whole juice. The remaining pulp (5.3 ml) was then diluted with an amount of diluent equivalent to serum removed, mixed, and assayed in the same manner as the whole juice. The results indicated that a large amount of pimaricin (105 μg/ml) was in the pulp fraction. With subsequent dilution, pimaricin becomes eluted from the pulp in some manner, allowing for its greater recovery. It should be noted that percent recovery in the serum fraction does not increase with dilution as does recovery in the pulp fraction, further indicating that the dilution-recovery effect is associated with the binding of pimaricin to the pulp fraction. In addition, the total recovery obtained by adding the serum and pulp recoveries was slightly greater than that obtained for the normal whole juice, indicating a possible method whereby pimaricin recovery in orange juice could be improved further.

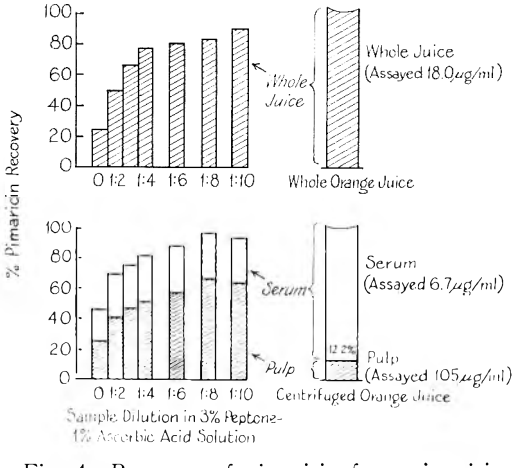

Fig. 4. Recovery of pimaricin from pimaricin-treated orange juice (20 μg/ml) and its recovery in the serum and pulp fractions of orange juice after centrifugation.

Further evidence that orange juice pulp binds pimaricin was demonstrated as follows: Fresh orange juice (15 ml) was centrifuged and the serum discarded. The pulp was mixed and washed four times in distilled water by recentrifuging and discarding the wash water each time. After the final centrifugation the water was decanted, leaving 1.2 ml of washed orange juice pulp, to which 13.8 ml of diluent containing 16 μg/ml of pimaricin was added and mixed. This whole sample (now containing pimaricin of 14.7 μg/ml) as well as the centrifuged pulp and serum fractions was assayed with increasing dilution as described previously. Prior to assay the pulp was resuspended in 13.8 ml of additional diluent. The assay results for the whole sample and serum and pulp fractions were respectively 13.4, 9.4, and 72 μg/ml of pimaricin, indicating that orange juice pulp removes pimaricin from the peptone-ascorbic acid solution. Thus, the dilution-recovery effect upon pimaricin assay in orange juice may be explained by a "binding effect" of pimaricin by the pulp fraction of the juice. The factor associated with the pulp that is responsible for binding pimaricin awaits further study.

REFERENCE

Shirk, R. J., and W. L. Clark. 1962. The effect of pimaricin in retarding the spoilage of fresh orange juice. Submitted to *Food Technol.*

Availability of Lysine in Wholewheat Bread and in Selected Breakfast Cereals

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SUMMARY

The protein in wholewheat unbaked bread ingredients, microwave-baked wholewheat bread, oven-baked wholewheat bread, and two breakfast cereals was evaluated by gain in body weight of rats, protein efficiency ratio, increase in carcass nitrogen, and apparent availability of lysine as determined by fecal excretion.

No significant differences were noted between the bread ingredients and microwave-baked bread. Oven-baked bread gave values significantly lower, 24–45%, than those of the ingredients. The apparent availability of lysine in bread ingredients and in microwave-baked bread was 96% and 95%, respectively, whereas that for oven-baked bread was 70%.

The protein in one breakfast cereal barely maintained the weight of the animals, and gave low values for protein efficiency ratio and increase in carcass nitrogen. Seventy-five percent of the lysine was available. The other cereal allowed good growth of the animals and gave values similar to those for nonfat milk. In this oat-and-wheat-germ cereal, 88% of the lysine was available.

A linear relation was obtained between either gain in body weight or increase in body nitrogen and the amount of available lysine ingested.

Heat has a detrimental effect on the protein in baked cereal mixtures. Morgan (1931) showed that the protein efficiency ratio and biological value of white bread were lower in the crust than in the crumb. She suggested that the disparity in nutritive value between toasted and raw products must lie chiefly in differences in the usefulness of the amino acid mixture absorbed, and that lysine and histidine were the amino acids probably involved. The protein efficiency ratio of a high-protein cake mixture was decreased by baking and toasting, and the ratio was restored by supplementing the toasted product with lysine (Block *et al.*, 1946). Two studies on high-protein (20–23%) biscuits made with peanut flour, corn flour, and other ingredients showed that available lysine was lost with baking (Mauron *et al.*, 1960; Carpenter and March, 1961).

Few studies have been made on the effect of heat on the availability of lysine in wheat products. Calhoun *et al.* (1960) found relatively small differences in the availability of lysine as determined by rat growth studies for wheat, white flour, and white bread: 72–76% available lysine in one experiment, and 78–83% in another. Since the bread contained nonfat milk solids, the protein was not exactly comparable with that of the flour.

The present study was designed to test whether the lysine in baked bread is as well utilized as is the lysine in the unfermented and unbaked ingredients. The apparent availability of the lysine was determined by the fecal excretion method of Kuiken and Lyman (1948) for unbaked wholewheat bread ingredients, microwave-baked bread, air-oven-baked bread, and 2 breakfast cereals. Apparent availability of lysine was compared with protein quality as determined by growth, protein efficiency ratio, and gain in carcass nitrogen per gram of nitrogen eaten.

EXPERIMENTAL

Protein efficiency ratios were determined by the rat-growth method of Osborne *et al.* (1919), with modifications. The rats used were the Long-Evans

strain, from the stock colony of the Department of Nutritional Sciences, University of California at Berkeley. They were weaned at 21 days, with weights between 50 and 60 g, and placed individually in metabolic cages. Animals were equally divided as to sex, weight, and litter mates for each group. They were then fed one of the 5 test diets *ad libitum* for 4 weeks. Food intakes and gains in body weights were recorded. Twenty animals in each group were fed a diet containing one of the breads, and 10 animals in each group were fed diets containing a breakfast cereal as the source of protein. A vitamin supplement was fed 3 times weekly. Animals received from the supplement calculated per day: thiamine HCl 20 μg, riboflavin 40 μg, pyridoxine 20 μg, calcium pantothenate 100 μg, niacin 66 μg, para-amino-benzoic acid 100 μg, inositol 2.5 mg, choline 10 mg, folic acid 20 μg, biotin 2 μg, menadione 49 μg, vitamin B_{12} 0.2 μg, vitamin A 100 I.U., vitamin D 10 I.U., and alpha-tocopherol 0.5 mg.

Lysine intakes and fecal excretions were determined for a 7-day period for all groups of animals. During the second week of the 4-week feeding period, feces were collected daily and refrigerated. At the end of that time they were weighed, ground, and frozen until analyzed for lysine. Samples of all diets for lysine analysis were finely ground and treated in the same manner as the fecal samples.

Baking of the breads. Bread was made from wholewheat flour containing 13.4% protein. The formula used contained, in parts: flour, 100; active dry yeast, 3.3; sucrose, 3.3; salt, 2.0; hydrogenated vegetable shortening, 1.9; water, 68.5. The ingredients were mixed in a large Hobart mixer, fermented 1 hr at 85°F, punched down, and fermented for 1 hr more.

For air-oven-baked bread, the dough was scaled to 20 oz, hand-molded into loaves, placed in tin bread pans of 11 × 4 × 2½ in., proofed for 1 hr, baked 45 min at 435°F in a hot-air oven, and cooled.

For microwave-baked bread, the dough was scaled to 14 oz, hand-molded into loaves, and placed in glass containers of 8 × 3½ × 2½ in. Because only one loaf of bread could be baked at a time, proofing was retarded by placing the loaves in a refrigerator, and the loaves were proofed to height. The loaves were baked 6.5 min in a microwave oven (Radar Range Model 1170, Raytheon), then turned at a 180-degree angle and baked for an additional 6.5 min. This turning gave more uniform baking.

The bread baked in the microwave oven differed from the bread baked in hot air in that the former had no visible browning or "crust" on the exterior of the loaf. Instead, a tough, rubbery skin covered the top of the loaf, and the sides and bottom were moist from the condensation of moisture forced to the exterior during baking. The crumb of the electronically baked bread was drier and harsher to the tongue than that of conventionally baked loaves, and the odor was less fragrant. The bread baked in the microwave oven also appeared to dry more rapidly when exposed to air. When loaves were baked for 15 min rather than for 13, hard, dry, dark-brown toast spots developed in the interior.

Composition of diets. The unbaked bread ingredients were prepared by thoroughly mixing the dry ingredients. The yeast was heated in an oven for ½ hr at 82°C to destroy its activity, and was pulverized before being mixed with the other ingredients.

The breads were thinly sliced, air-dried for 48 hr at room temperature, ground finely, and refrigerated until used.

The flaked, ready-to-eat cereal (Kellogg's Special K, no added lysine; Kellogg Co., Battle Creek, Michigan) was ground before being incorporated into the diet. The product was advertised as being a high-protein cereal composed of rice, wheat gluten, wheat germ, nonfat dry milk, and dried yeast. It contained 2.75% of nitrogen.

The granular uncooked cereal (Protein Plus; General Mills, Inc.), containing oats and toasted wheat germ, was used as purchased. This was advertised as a high-protein hot oat cereal providing high-quality protein. The sample contained 2.90% of nitrogen.

The bread and cereals to be tested were incorporated into diets made to a nitrogen content of 1.65±0.04%. The composition of the diets is shown in Table 1.

Carcass analysis for nitrogen. At the end of the 4-week feeding period the animals were fasted for 24 hr. They were then killed with ethyl ether, weighed, wrapped in aluminum foil, and frozen until analyzed. Intestinal tracts were included. Incisions were made into the skull and the thoracic and body cavities, and the carcasses were dried 48 hr at 65°C in a vacuum oven.

The dried carcasses were extracted with ethyl ether for 24 hr, in a Soxhlet apparatus to avoid the foaming that occurs during digestion in the Kjeldahl analysis for nitrogen. Each dried, defatted carcass was digested with concentrated sulfuric acid in the proportion of 10 ml/g dried carcass. Selenium and Cu_2SO_4 were used as catalysts; 2-octanol was used to control the frothing. The digested matter was diluted to 1 L, and aliquots were taken for the micro-distillation of ammonia.

A group of 10 stock rats, weighing an average of 45 g, was analyzed for carcass nitrogen and found to contain 2.77±0.06% nitrogen. This value

Table 1. Composition of diets (percentages).

Diet	Protein source	Confectioner's sugar	Salt mix XIV [a]	Hydrogenated shortening	Nitrogen
A. Unbaked wholewheat bread ingredients	70	15	5	10	1.69
B. Microwave-baked wholewheat bread	68	17	5	10	1.64
C. Air-oven-baked wholewheat bread	72	13	5	10	1.63
D. Ready-to-eat cereal	58	27	5	10	1.68
E. Cereal to-be-cooked	56	29	5	10	1.63

[a] General Biochemicals, Inc., Laboratory Park, Chagrin Falls, Ohio.

was used as the amount of carcass nitrogen present at the beginning of the experiment.

Determination of lysine. Five-mg samples (25–50 μg lysine) were weighed into Pyrex glass tubes, and 0.5 ml of constant-boiling, glass-distilled HCl was added. Air was evacuated, the tubes sealed, and the contents hydrolyzed for 6 hr at 110°C according to the method of Levy and Chung (1953). The tubes were opened, and the acid was evaporated in a desiccator over NaOH flakes. Distilled water was added, and the samples were redried to remove all traces of acid. One-tenth ml of distilled water was added to dissolve the amino acids in the sample, and duplicate 10-μl portions were applied to strips of Whatman No. 1 filter paper washed in 95% ethanol to remove all traces of ammonia. The samples were developed for 12 hr in a solvent system of *n*-butanol–glacial acetic acid–distilled water, 4:1:5. The chromatogram strips were dried for 20 min in forced air at 65°C, sprayed with alkaline ninhydrin, and redried for 20 min for maximum color development. The lysine spots were cut from the paper strips and placed in 15-ml centrifuge tubes. Blanks were cut from the paper where no amino acids had been run. Five ml of 71% ethyl alcohol were pipetted into the tubes, which were sealed with parafilm, shaken, and centrifuged 3 min at 2400 rpm. The eluate was read in a Beckman B spectrophotometer at 575 mμ, sensitivity 4, and the results were interpolated from a standard curve (Kay *et al.*, 1956).

RESULTS

Gain in body weight. Of the animals fed bread diets, those fed the unbaked wholewheat bread ingredients, diet A, showed the largest average gain in body weight; those fed the air-oven-baked wholewheat bread, C, had the lowest gain (Table 2). Differences between all 3 groups were highly significant by the *t* test (Table 5). Animals fed the ready-to-eat cereal diet barely maintained their initial weight. The animals fed the cereal to-be-cooked were the largest of the groups, with growth approaching that shown by animals fed a nonfat dry milk diet containing 10% of protein (Kennedy and Sabiston, 1960). Weight gains paralleled food intakes.

Protein efficiency ratios. The protein efficiency ratio of the microwave-baked wholewheat bread was 5% lower than that of the unbaked ingredients, and the ratio for the air-oven-baked bread was 24% lower (Tables 2 and 5). The difference between the unbaked ingredients and the micro-

Table 2. Body weight gains and protein efficiency ratios.[a]

Diet	Nitrogen intake (g)	Gain in body wt. (g)	Gain in body wt. Std. dev.	Protein efficiency [b] Ratio	Protein efficiency [b] Std. dev.
A. Unbaked wholewheat bread ingredients	4.41	43.4	7.4	1.68	0.15
B. Microwave-baked wholewheat bread	3.68	34.5	7.2	1.60	0.16
C. Air-oven-baked wholewheat bread	3.15	23.5	4.8	1.28	0.22
D. Ready-to-eat cereal	1.56	3.3	1.2	0.34	0.12
E. Cereal to-be-cooked	5.41	96.2	10.1	2.84	0.17

[a] Four-week period; 20 animals per group in A, B, and C, and 10 in D and E. Diets contained 1.65 ± 0.04% N.
[b] G weight gain/g protein intake.

Table 3. Increase in carcass nitrogen.

Diet	Carcass nitrogen at beginning of experiment (2.77% of body weight) (g)	Carcass nitrogen at autopsy			Gain in carcass nitrogen	
		(%)	Std. dev.	(g)	Total (g)	Per g N eaten (mg)
A. Unbaked wholewheat bread ingredients	1.80	2.76	0.16	2.98	1.18	268
B. Microwave-baked wholewheat bread	1.85	2.80	0.13	2.83	0.98	266
C. Air-oven-baked wholewheat bread	1.82	2.65	0.19	2.39	0.57	181
D. Ready-to-eat cereal	1.37	2.96	0.13	1.57	0.20	128
E. Cereal to-be-cooked	2.00	2.78	0.11	4.69	2.69	497

wave bread was not significant by the *t* test, whereas the differences between either of these groups and the air-oven-baked bread were highly significant. The protein efficiency ratio for the ready-to-eat cereal was low, 0.3; that of the cereal to-be-cooked was 2.8.

The use of male rats has been suggested for more uniform response in protein efficiency ratios (Derse, 1960; Morrison and Campbell, 1960) although Morgan (1931) noted no difference in ratios between male and female rats. Protein efficiency ratios for 10 females on each of diets A, B, and C were slightly higher (4–9%) than for an equal number of males, but the differences were not statistically significant.

Diet	Protein efficiency ratios and standard deviation		*t* value
	Females	Males	
A	1.73 0.13	1.64 0.16	1.22
B	1.63 0.15	1.56 0.16	0.97
C	1.34 0.25	1.22 0.18	1.16

Carcass nitrogen. For all groups the increase in carcass nitrogen (Table 3) followed the trends given by the gains in body weight. The relationships between increase in nitrogen per g of nitrogen eaten were similar to those for protein efficiency ratios—there was no appreciable difference between the unbaked ingredients and the microwave-baked bread, and the values for air-oven-baked bread were lower than those for either.

No significant differences in percent carcass nitrogen were shown among groups A, B, C, and E. The highest value, 2.96%, was found in the animals fed diet D, the ready-to-eat cereal. Carcasses of these animals had a significantly higher percentage of nitrogen—the *t* value when comparing groups D and E was 4.86. These animals barely maintained their initial weight during the experiment, and the carcasses showed very little visible fat. This increased concentration of protein in the carcasses of rats fed a restricted-calorie diet was noticed by Rosenthal and Allison (1956). They stated that the increase was probably due to reduction of body fat and that, when calculated on a fat-free basis, the protein was constant regardless of caloric intake on either protein-free or 5% casein diets.

Lysine excretion. Table 4 shows the amount of lysine excreted in the feces on the test diets, as

Table 4. Lysine excretion and availability.

Diet	No. of animals	Lysine in diet per g: Nitrogen (g)	Diet (mg)	Lysine intake (7 days) (mg)	Lysine excretion (7 days) (mg)	Lysine excreted (%)	Std. dev. (%)	Lysine apparently available
A. Unbaked wholewheat bread ingredients	20	0.31	5.2	355	14.6	3.9	4.3	96
B. Microwave-baked wholewheat bread	20	0.32	5.2	309	14.7	4.9	3.3	95
C. Air-oven-baked wholewheat bread	20	0.31	5.0	247	73.6	30.5	27.1	70
	17 [a]			250	50.7	20.5	3.1	80
D. Ready-to-eat cereal	10	0.20	3.4	69	18.9	25.8	18.7	74
E. Cereal to-be-cooked	10	0.61	10.0	908	108.1	12.1	7.0	88

[a] Average of 17 animals, omitting three that showed very high excretions.

Table 5. Comparison of quality of protein in bread and cereals by various methods.

Diet	No. of animals	Body weight			Protein efficiency			Increase in carcass nitrogen		Lysine apparently unavailable	
		Gain (g)	Decrease from A (%)	t value	Ratio	Decrease from A (%)	t value	(mg/g N eaten)	Decrease from A (%)	(%)	t value
A. Unbaked wholewheat bread ingredients	20	43.4	....	....	1.68	....	....	268	....	3.9	....
B. Microwave-baked whole-wheat bread	20	34.5	20.5	A,B 3.86 [a]	1.60	5.2	A,B 1.73	266	0.7	4.9	0.83
C. Air-oven-baked whole-wheat bread	20	23.5	44.9	A,B 10.06 [a] B,C 5.69 [a]	1.28	23.8	A,B 6.62 [a] B,C 5.51 [a]	181	32.4	30.5	A,B 4.34 [a] B,C 4.19 [a]
	17									20.5	A,B 13.3 [a]
D. Ready-to-eat cereal	10	3.3			0.34			128		25.8	
E. Cereal to-be-cooked	10	96.2			2.84			497		12.1	

[a] Significant at the 1% level.

related to the lysine intake. The 20 animals fed the unbaked wholewheat-bread diet excreted an average of 3.9% of the lysine ingested. Fourteen of these animals excreted 1.63±0.15% of the ingested lysine, so that most of the variability occurred in 6 of the animals. The animals fed the microwave-baked bread diet excreted slightly more of the lysine, 4.9%, an increase not statistically significant when compared with the whole group fed the unbaked ingredients. Animals fed the air-oven-baked bread diet, however, excreted 30.5% of the ingested lysine. Three of the animals on this diet showed high excretion as compared with others in the group. Excluding these 3 animals, the remainder excreted an average of 20.5% lysine, a highly significant difference when compared with excretion on either of the other bread diets. Animals fed the ready-to-eat cereal excreted a higher percentage of the ingested lysine (25.8%) than did those fed the raw-oat and toasted-wheat-germ cereal (12.1%).

DISCUSSION

Evaluation of protein quality. The protein quality of the air-oven-baked wholewheat bread was significantly lower than that of the unbaked ingredients by all criteria used in this study—gain in body weight, protein efficiency ratio, increase in carcass nitrogen per g of nitrogen eaten, and apparently available lysine (Table 5). The decreases ranged from 24 to 45%.

Fermentation and baking in a microwave oven did not significantly affect the protein of the ingredients. The protein quality of microwave-baked bread, diet B, was slightly lower (1–5%) than that of the unbaked ingredients, diet A, by all of the tests except gain in weight, but none of the differences was statistically significant. Body weight gains of the animals fed diet B were 20% lower than those of the animals fed diet A—a statistically significant decrease. However, the animals fed diet B ate less food than those fed diet A. The protein quality of the air-oven-baked bread was significantly lower (20–32%) than that of the microwave-baked bread by all methods used.

All of the methods indicated low protein quality of the ready-to-eat cereal. Animals fed this cereal barely maintained their weight during the course of the experiment. The protein efficiency ratio and the gain in carcass nitrogen were low, and of the lysine

ingested, about ¼ was apparently not available.

The cereal to-be-cooked had the highest-quality protein of any of the foods tested when judged by gain in body weight, protein efficiency ratio, and gain in carcass nitrogen. The protein efficiency ratio approached that of milk (Kennedy and Sabiston, 1960). About 12% of the lysine in this raw oat–wheat germ cereal was apparently unavailable. Peters *et al.* (1947) found that 7.2% of the lysine ingested was excreted by rats fed a diet containing raw oats, and 22.5% when exploded oats were fed.

Comparison of methods. With the exception of the group of animals fed diet B, gain in body weight was a good index of protein quality. The rats fed diet B ate less food, and so gained less weight, although by all other criteria the protein quality was similar to that of diet A. These data show that, while in many cases increase in body weight alone is a good index of protein quality, exceptions do occur because of variation in food intake, which should always be taken into consideration.

In all groups the increase in carcass nitrogen per g of nitrogen eaten gave no better evaluation of the protein quality than did the protein efficiency ratio, and the determination was more time consuming. Determination of variability within groups, needed for statistical analysis of significant differences among groups, is not possible since, of necessity, carcass nitrogen at the beginning of the experiment must be estimated, not determined, from average values for percent body nitrogen.

For the five products studied, a linear relationship was shown when either gain in body weight or increase in carcass nitrogen was plotted against the amount of available lysine consumed (Fig. 1). This is in agreement with data of Calhoun *et al.* (1960).

Availability of lysine. Apparent availability of lysine was about 20–30% less in oven-baked wholewheat bread than in the ingredients, and little difference was found between the ingredients and microwave-baked bread. The apparent availability of lysine was calculated from fecal excretion and lysine intake. Fecal excretion was not corrected for endogenous lysine excretion. Kuiken and Lyman (1948) stated that the correction to be applied to apparent availability, in order to give true availability, was quite small. According to their data, true availability of lysine in roast beef was about 4% higher than apparent availability.

Calhoun *et al.* (1960) found fecal excretions of lysine from wheat, flour, and bread to be respectively 26, 31, and 9% on a gluten basal diet, and those from wheat, flour, bread, and gluten to be 24, 8, 17, and 1% on an amino acid basal diet. Diets contained 70% wheat, flour, or bread, or 20% gluten. Total daily lysine excretion (13–90 mg) was much higher on the wheat diets than in the present study (2 to 15 mg), where the diets contained 56–70% of the test protein. Calhoun *et al.* stated that they did not obtain uniformly good agreement between available lysine from fecal excretion and from rat-growth studies. From the rat-growth studies Calhoun *et al.* found little difference in lysine availability between flour and bread: respectively 75, 72, and

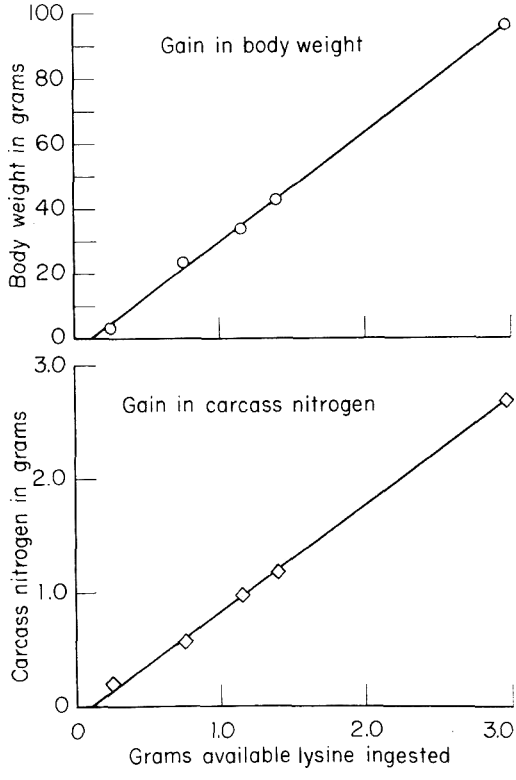

Fig. 1. Gain in body weight and gain in carcass nitrogen versus available lysine ingested.

76% availability in wheat, flour, and bread on a gluten basal diet, and 78, 80, 83, and 80% in wheat, flour, bread, and gluten on an amino acid basal diet. Perhaps the method of determining lysine could account for some of the differences between the studies, since microbiological assays were used by Calhoun *et al.* and chromatographic determinations were used in the present work.

Clegg and Davies (1958), using chemical methods, reported that the ratio of "available lysine" to total lysine in bread crumbs was not significantly different from that in white flour. A reduction of 15% in "available lysine" was found in the crust.

Effect of heat. Peters *et al.* (1947) showed that the only essential amino acid affected to any extent in heat-processed oat protein was lysine. Their work was based on microbiological amino acid assays of samples hydrolyzed with enzymes *in vitro*. In addition, animal-feeding experiments showed that the lysine was less available *in vivo*. The present study shows that these latter findings on oats can also be demonstrated in wheat.

The lowered protein quality in the air-oven-baked bread, as evinced by decreased protein efficiency ratio and gain in carcass nitrogen, was associated with a decrease in the apparent availability of lysine, whereas in the microwave-baked bread, which had a protein quality similar to that of the unbaked ingredients, the lysine was nearly as available as in the ingredients. Thus, the lowered quality of the protein in oven-baked bread could have been due, at least in part, to the decrease in availability of the lysine. A small amount of lysine in the protein of bread dough has been found to be destroyed by baking in hot air—4% in this study, 2–16% as reported by Rosenberg and Rohdenburg (1951), and 2–10% as reported by Pomeranz (1962). McDermott and Pace (1957) reported 3% less total lysine in bread crumb, and 14% less in bread crust, than in flour, where the crust was 18% of the loaf. Of the lysine that remains in the bread, only part is apparently available. Thus, the unavailability of the lysine is in addition to its actual destruction. The data of Calhoun *et al.*, however, showed no difference in availability of lysine between baked bread and the uncooked wheat and flour.

The decreased protein quality of the air-oven-baked bread may have been due both to the length of time that it was exposed to heat and to the intensity of the heat. The shorter baking time in the microwave oven—13 min, compared with 45 min in the hot-air oven—and the presence of a tough outer skin without browning in the microwave-baked bread, were indications of less severe heat treatment.

Based on criteria of protein quality in the present study, the data indicate that the ready-to-eat cereal (Diet D) might have received excessive heat treatment in processing. This cereal, consisting of a mixture of rice, wheat gluten, wheat germ, nonfat milk, and yeast, had an appreciable amount of lysine that apparently was not available.

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The conclusions are not affected, but an error in calculating the concentration values of acetic acid resulted in erroneous difference thresholds (by a factor of 10^3). Thus, all values reported as $\times 10^{-5}$ should read $\times 10^{-2}$, as follows: p. 452, four places in Summary; p. 453, last sentence; p. 454, 1st sentence; and p. 455, fig. 2 Y axis, Table 4 subheadings, and last sentence in Experimental Results.

RESEARCH NOTE

A New Method for Enumerating Bacteria in Frozen Egg with Special Reference to the Transmission of *Borrelia anserina* [a]

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This study was initiated as the result of certain data obtained in direct microscopic examination of frozen and liquid egg products that are important components of many frozen foods. Frozen eggs of good quality handled under excellent processing conditions will not contain more than one microorganism per 5–20 fields. In the preliminary studies, numbers were varied and frequently fantastically high, meriting additional study. A rapid volumetric method of measuring egg to the microscope slide is normally used (American Public Health Association, 1958, 1960), but the method contains well known defects. If the pipettes used are small enough to measure 0.01–0.10 ml accurately to the slide, the amount retained in the pipette is variable and the volume-to-weight ratios are not necessarily reproducible.

In the studies made in these laboratories, weighing a 0.01–g sample replaces the volumetric procedure. If microscope slides are carefully cleaned, and numbered with a diamond-point pencil, they may be used repeatedly from desiccator storage. The sample is spread with a needle over the area of 1 sq cm, dried under a lamp, defatted in xylol, and stained in methylene blue by usual procedures. The counts obtained were higher with this technique than with the Broadhurst Paley stain. The results are highly reproducible.

A number of samples of egg of low quality were obtained, in certain instances accompanied with large numbers of tissue cells. One sample of frozen egg yolk, supposedly of good quality, was found to contain more than 50 *Borrelia* per field and a large number of tissue cells. In addition to indicating the presence of incubator rejects in this sample it has profound significance in that the organisms correspond to *Borrelia anserina,* responsible for spirochetosis in fowl. In the past this disease has been assumed to be transmitted by tick and mite biological vectors, and in a number of outbreaks in which these were absent the transmission has been unexplained. Our laboratories are now investigating the frequency of these organisms in eggs and whether in certain instances the chicks infected with *Borrelia* might survive and introduce the infection into a flock previously free of the organisms.

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