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CONTENTS

Volatile Chemical Compounds in Dry-Cured Hams[a, b]

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(Manuscript received March 11, 1963)

SUMMARY

Volatile compounds isolated from dry-cured hams were tentatively identified by gas chromatography retention times and further verification of the compounds made by infrared spectroscopy. These compounds were as follows: formaldehyde, acetaldehyde, propionaldehyde, isobutyraldehyde, *n*-valeraldehyde, isovaleraldehyde, acetone, diacetyl, methyl ethyl ketone, formic acid, acetic acid, propionic acid, butyric acid, and isocaproic acid. R_F values and selective indicators were used to identify ammonia and methylamine. Selective trapping was used to identify hydrogen sulfide and trace amounts of disulfides and/or monosulfides.

INTRODUCTION

The commercial economic importance of dry-cured country-style hams (hereinafter referred to as CS hams) is increasing in the Southeastern United States. This type of ham was described by Dunker and Hankins (1951).

The acceptability of CS hams is determined by odor and flavor, among other quality factors. A few studies have attempted to relate odor and/or flavor to specific chemical constituents (Besley and Carroll, 1942; Blumer, 1954, 1958; Brady *et al.*, 1949; Cecil and Woodroof, 1954; Howe and Barbella, 1937; Hunt *et al.*, 1939; Kemp *et al.*, 1957, 1961).

Other studies of CS hams related to aroma and flavor have been conducted primarily by subjective methods. These methods may indicate the range of acceptability, but they are not precise enough to measure accurately the effect of treatments on the development of aroma and flavor. The aroma from CS hams is distinctive and resembles the flavor; therefore, it seemed reasonable to assume that the determination of volatile constituents is important for objective evaluation of these quality criteria. This study was initiated to determine methods satisfactory for the isolation, separation, and identification of volatile compounds in dry-cured CS hams under prescribed experimental conditions.

MATERIALS AND METHODS

Curing and aging of hams. Sixty-four regular short-cut skinned hams, weighing 14–16 lb, were cured for 2 days per lb at 4°C with 1 oz of curing mixture (8 lb NaCl, 2 lb white sucrose, and 3 oz potassium nitrate) per pound of ham. One-third of the total quantity required for each ham was applied to the surface on each of the 1st, 3rd, and 10th days. After curing, the hams were soaked 2 hr in cold water, dried, and then smoked at 21°C by burning hardwood sawdust. They were aged at 23±1°C, relative humidity 60±3%, and air flow 35 ft/min. Hams were sampled after they were cured and also after they were smoked. They were sampled during aging at monthly intervals for 6 months, and after 9, 12, 15, and 24 months. One ham was randomly selected for analysis at each of the sampling periods.

The NaCl and moisture content of hams will be quite uniform after curing 2 days/lb and storing for an additional 30 days.

Vacuum distillation. A 400-g portion of a 1-in.-thick cross-sectional center-cut ham slice was freed of subcutaneous fat, ground in a Waring blender and placed in a sample flask, and the volatile compounds were separated by vacuum dis-

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[b] This work is cooperative with the U. S. Department of Agriculture.

[c] From a portion of a Ph.D. thesis, North Carolina State College, 1962.

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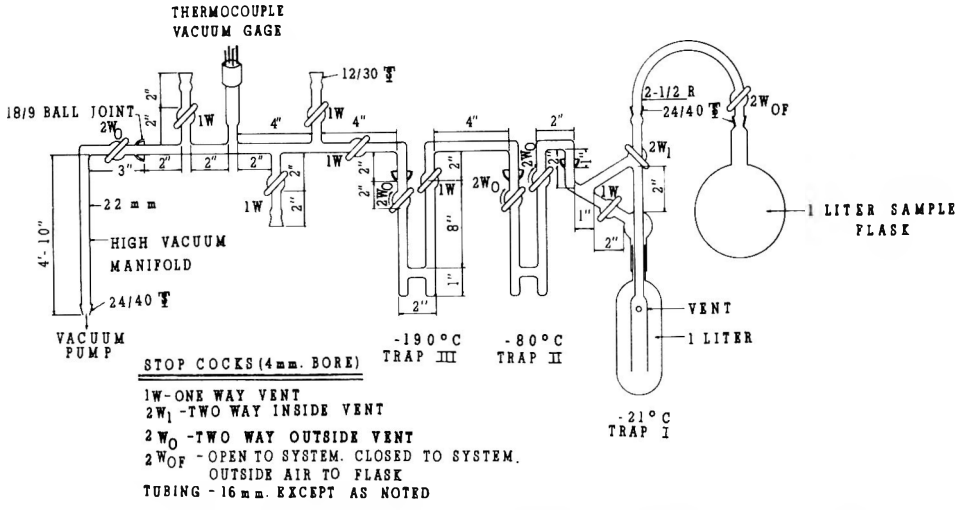

Fig. 1. Vacuum distillation apparatus (modification of Niegisch and Stahl, 1956).

tillation (Fig. 1). The sample was distilled 24 hr at 60°C under a vacuum of 200 μ. Typical aroma and flavor were developed under these conditions. Traps, arranged in order of decreasing temperature, were cooled with ice-salt, dry ice-methanol, and liquid nitrogen. After distillation, the contents of the traps were poured or bubbled into appropriate trapping solutions as described below. The odor after distillation was considered nearly neutral when the odor of the residue was compared with the odor of cooked portions of similar hams. Thus, it appeared that the distillation procedure for removal of volatile constituents was satisfactory. However, it is conceivable that higher distillation temperatures would produce compounds different from those produced here.

Trapping the volatile carbonyl compounds. Volatile carbonyl compounds were trapped in a solution containing 2 g 2,4-dinitrophenylhydrazine (DNP) per liter of 2*N* hydrochloric acid. The resulting 2,4-dinitrophenylhydrazones were collected by filtration and dried, first in air and then in a vacuum desiccator over phosphorus pentoxide.

Micro quantities of other carbonyl compounds, if present, may not have been detectable by the above technique.

Volatile carbonyl compounds from hardwood smoke were also trapped in DNP solution by pulling the smoke with a vacuum through a gas washing bottle containing the solution.

Gas chromatography of volatile carbonyl compounds. The carbonyl derivatives were analyzed by gas chromatography using a modification of the method of Ralls (1960a). An approximate 10-mg portion of a mixture of the hydrazones and alpha-ketoglutaric acid (1:3 by wt) was introduced into a Pyrex capillary tube sealed at one end, and prepared for injection into a gas chromatography (GC) apparatus (Fig. 2). The parent carbonyl compounds were regenerated and swept into the GC apparatus (Model 154-B Vapor Fractometer, Perkin-Elmer Corp.) by heating the capillary tube to 250°C in 15 sec and maintaining this temperature for an additional 15 sec.

The column (2 m × 6.35 mm) used to separate the carbonyl compounds was packed with Celite coated with diisodecyl phthalate, and maintained at 82°C. The carrier gas (helium) pressure in the column was regulated at 10 lb/sq in. and gave a flow rate of 32 ml/min (Woods, 1960).

To recover the separated carbonyl compounds, the tip of a glass tube attached to the exit port of the gas chromatograph was immersed in 5 ml of DNP solution whenever the recorder response indicated that a carbonyl compound was being eluted from the column. Several runs were made to obtain a sufficient quantity of hydrazone for subsequent identification by infrared spectroscopy. Each hydrazone was centrifuged from solution, washed with 2*N* HCl and then with distilled water, and dried over phosphorus pentoxide.

Infrared analysis of 2,4-dinitrophenylhydra-

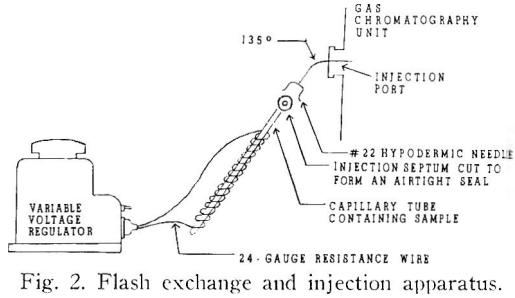

Fig. 2. Flash exchange and injection apparatus.

zones. The dried hydrazone was mixed with potassium bromide (0.5:99.5) and placed in an evacuable potassium bromide die (Research and Industrial Instrument Co.). Vacuum was applied to the die for 15 min, and the die, still under vacuum, was placed in a press (17,000 lb/sq in.) for 3 additional minutes. The pellet thus formed was placed in the sample beam of a double beam Model 137 Infracord Spectrophotometer (Perkin-Elmer Corp.) and the spectrum recorded. The spectrum was compared with 2,4-dinitrophenylhydrazones of known carbonyl compounds (Eastman Chemical Co.) purified by gas chromatography as described above. Thus, known compounds from different homologous series could be differentiated as well as compounds having different chemical groups.

Trapping the volatile acids. Short-chain volatile acids were trapped in 1*N* potassium hydroxide solution. The potassium salts thus formed were collected over a steam bath and dried in a vacuum oven at 100°C.

Gas chromatography analysis of volatile acids. The method of Ralls (1960b) was used in the GC analysis of the volatile acids. The potassium salts of the acids were heated with potassium ethyl sulfate to form the ethyl esters (Evans and Albertson, 1917). The esters were separated by a procedure similar to that described above for the carbonyl compounds, except that the injection capillary temperature was raised to 300°C and the column temperature to 120°C.

As the individual ethyl esters emerged from the GC apparatus, they were trapped in a U-tube submerged in liquid nitrogen. The esters were then warmed to room temperature and dissolved in carbon tetrachloride (CCl_4).

Infrared analysis of esters. An aliquot of a solution of each ester in CCl_4 (5:95) was placed in a sodium chloride cell, and the infrared spectrum was recorded with CCl_4 the blank in the reference beam.

Trapping of volatile basic compounds. The ham samples were steam distilled at atmospheric pressure, and the volatile basic compounds were passed into a cold trap (ice and salt). The noncondensable gas was then bubbled through 1*N* HCl. The trapped components were combined and dried on a steam bath, and the drying was completed in a vacuum oven at 100°C.

Paper chromatography of volatile basic compounds. The hydrochlorides of the volatile basic compounds were separated by ascending paper chromatography by the method proposed by Davies *et al.* (1953), as modified by Hornstein *et al.* (1960) and Hornstein (1960). Whatman No. 1 paper was used as the stationary phase. The chromatogram was developed in a test tube (25 × 250 mm) for 90 min.

Selective trapping of volatile sulfur compounds. A slurry was made by blending 600 g of ham with 300 ml of distilled water. A sulfur absorption train as described by Dateo *et al.* (1957) and Hasselstrom (1957) was used to trap the volatile sulfur components. Refluxing and sweeping with nitrogen (30 ml/min) was continued for 5½ hr.

RESULTS AND DISCUSSION

Carbonyl compounds in hams. Fig. 3 is a representative chromatogram of the carbonyl compounds from one ham. The retention times for known carbonyl compounds are given in Table 1. From these values, the peaks of the chromatogram in Fig. 3 were tentatively identified.

For a more positive identification, the compounds labeled in Fig. 3 as acetaldehyde, propionaldehyde, isobutyraldehyde, diacetyl, 2-butanone (methyl ethyl ketone),

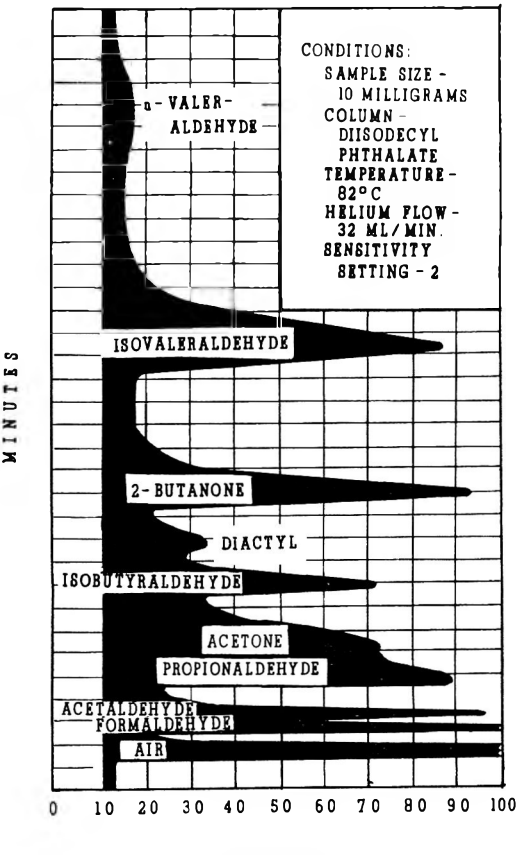

Fig. 3. Gas chromatogram of volatile carbonyl compounds isolated from a ham aged six months.

Table 1. Retention times of carbony compounds regenerated from 2,4-dinitrophenylhydrazones by exchange with alpha-ketoglutaric acid.

Parent carbonyl	Retention time (min) [a]	Retention volume (ml) [b]
Formaldehyde	1.4	45
Acetaldehyde	2.2	70
Propionaldehyde	4.0	128
Acrolein	4.2	134
Acetone	4.7	150
Isobutyraldehyde	7.7	246
n-Butyraldehyde	11.4	365
Diacetyl	11.5	368
Methyl ethyl ketone	11.8	378
Methyl isopropyl ketone	16.8	538
Isovaleraldehyde	17.8	570
Crotonaldehyde	20.5	656
Aldol	21.0	672
Methyl *n*-propyl ketone	21.5	688
Diethyl ketone	22.2	710
n-Valeraldehyde	25.7	822

[a] Conditions: 2-meter column of diisodecyl phthalate, temperature of 82°C, and a helium flow of 32 ml/min.

[b] Retention volume = retention time × flow rate (32 ml/min.).

and isovaleraldehyde were trapped in the DNP solution as they emerged from the gas chromatograph, after which they were subjected to infrared spectroscopy. Excellent agreement was found between different classes of the known and sample compounds. However, the spectrum of a known sample of diacetyl was similar to the spectrum produced by a 75:25 mixture of diacetyl and isobutyraldehyde. It is therefore probable that the "diacetyl" peak of the sample contained some isobutyraldehyde. Other investigators have reported the following volatile carbonyls in meat: acetaldehyde, Pippen *et al.* (1958), Gaddis *et al.* (1959), Gaddis and Ellis (1959), Landmann (1960), Hornstein (1960), and Kramlich and Pearson (1960); propionaldehyde, Pippen *et al.* (1958), Gaddis *et al.* (1959), and Gaddis and Ellis (1959); diacetyl, Pettet and Lane (1940) and Pippen *et al.* (1958); and 2-butanone, Gaddis *et al.* (1959) and Pippen *et al.* (1958).

Attempts failed to trap the gas corresponding to formaldehyde (Fig. 3) and produce the hydrazone derivative. This result may have been due to the highly volatile nature of the gas.

Other carbonyl compounds listed in Fig. 3 were found in trace amounts only; therefore, no further identification was made. However, acetone has been reported in pork fat by Gaddis *et al.* (1959), in chicken by Pippen *et al.* (1958), and in beef and pork by Landmann (1960) and Hornstein (1960). Callow (1927) identified formaldehyde in smoked meats, and Hornstein (1960) listed it as a constituent of beef.

Wood smoke was analyzed, and peaks were recorded with retention times characteristic of formaldehyde, acetaldehyde, acetone, methyl ethyl ketone, isovaleraldehyde, and *n*-valeraldehyde. Pettet and Lane (1940) also isolated formaldehyde, acetaldehyde, and acetone; however, they also found diacetyl, which was not identified with the procedure used here. Callow (1927) also found formaldehyde to be present in wood smoke.

The ratios among the carbonyl compounds as determined by gas chromatography remained quite constant during the entire aging period, except for 2-butanone. For this compound, the proportion appeared to increase during the latter part of the aging period. Although the ratios remained constant, the total quantity of carbonyl derivatives obtained from the hams increased with aging time. This agrees with the results of Pippen *et al.* (1958), Gaddis and Ellis (1957), Gaddis *et al.* (1959), Hornstein (1960), and Mendelsohn and Steinberg (1962), who found that conditions favorable to oxidation gave an increase in carbonyl compounds.

Whether or not a quantitative relationship exists between flavor and carbonyl compounds is not reported in this paper.

Volatile acids in hams. Fig. 4 is a representative chromatogram indicating the volatile acids (esters) obtained from a ham aged 9 months. These acids were identified by comparison with chromatograms of known acids, retention times for which are shown in Table 2. The acids found in the hams were tentatively identified as formic, acetic, propionic, butyric, and isocaproic.

Sufficient quantities of acetic and butyric acids were trapped to allow comparison of their infrared spectra with those of known

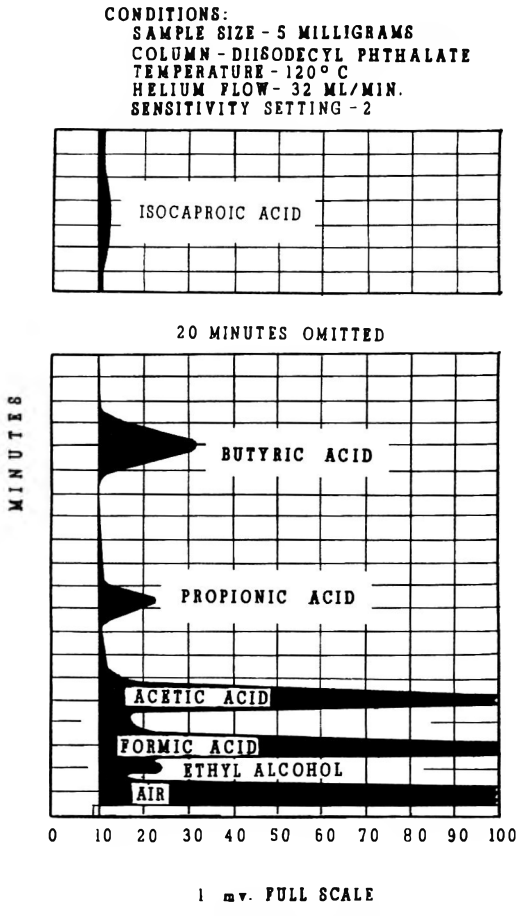

Fig. 4. Gas chromatogram of volatile acids isolated from a ham aged nine months.

acids. Excellent agreement was found; thus, verification of the same chemical grouping was shown. Acetic acid was isolated from CS hams by Blumer (1954). The other acids mentioned above could not be isolated in quantities sufficient to form derivatives. Only one peak was obtained from the smoke analysis having a retention time the same as that of the formic ester.

The number and quantity of acids isolated were found to increase with the length of the aging period, except for the quantity of formic acid. The percentage of this acid decreased with storage time, but the relative decrease was probably due to an increase of longer-chain acids rather than to a decrease in amount of formic acid. It should be mentioned at this point, however, that formic acid is a constituent of wood smoke, and some decrease of the acid from this source

Table 2. Retention times for ethyl esters of volatile acids.

Parent acid	Retention time (min) [a]	Retention volume (ml) [b]
Ethyl alcohol [c]	1.9	61
Formic	2.4	77
Acetic	4.3	138
Acrylic	7.7	246
Propionic	7.9	253
Isobutyric	10.3	330
Trimethylacetic (pivalic)	11.8	378
Methacrylic	12.5	400
Butyric	14.2	454
Isovaleric	20.4	653
Crotonic	21.5	688
Valeric	27.5	880
Dimethylacrylic	36.7	1174
Isocaproic	39.7	1270
Caproic	53.3	1706

[a] Conditions: 2-meter column of diisodecyl phthalate, temperature of 120°C, and a helium flow of 32 ml/min.

[b] Retention volume = retention time × flow rate (ml/min).

[c] Ethyl alcohol is sometimes a disintegration product of the ester formation, thus forming a peak on the gas chromatography tracing.

may have occurred, especially since the residual of wood smoke is deposited primarily upon the meat surface.

Volatile bases in hams. Table 3 shows the paper chromatography results for known amine hydrochlorides. Comparison of these values with those obtained from the spots on the chromatogram from the ham samples indicates that the major portion of the volatile bases was ammonia and a very small amount was methylamine. This is in agreement with the results reported by Hornstein *et al.* (1960) and Hornstein (1960) for beef, and with the basic components of meat flavor as listed by Landmann (1960); however, Landmann also noted the presence of ethylamine, which was not detected in this study. Pippen and Eyring (1957) found only ammonia in chicken broth.

Volatile sulfur compounds in hams. Almost immediately upon refluxing and sweeping with nitrogen, lead sulfide precipitate was noted in the solid lead acetate trap, indicating that hydrogen sulfide had been liberated from the ham under these conditions. Several research workers have reported the presence of hydrogen sulfide in extracts of

Table 3. Paper chromatography separation of known amine hydrochlorides.

Compound	Rf	Ninhydrin test	Methyl orange test
NH_4Cl	0.50	Negative	Positive
$CH_3NH_2 \cdot HCl$	0.45	Positive	Positive
$CH_3CH_2NH_2 \cdot HCl$	0.53	Positive	Positive
$CH_3(CH_2)_2NH_2 \cdot HCl$	0.60	Positive	Positive
$CH_3(CH_2)_3NH_2 \cdot HCl$	0.68	Positive	Positive
$(CH_3CH_2)_2NH \cdot HCl$	0.78	Negative	Positive

meats (Crocker, 1948; Bouthilet, 1951a,b; Pippen and Eyring, 1957; Hornstein, 1960). A 3½-hr period of continuous refluxing followed before a trace of other precipitates was noted in the aqueous mercuric chloride traps. Thus, with a longer refluxing time, a trace of disulfides and/or monosulfides was obtained. Under the conditions of this experiment no mercaptans were detected.

The methods of analysis used in this study appear to be, in general, adequate for determining the volatile compounds in CS hams. Since these hams were aged for long periods, some differences in the amount and kind of these compounds were noted at the several sampling periods. Some association may exist between aged flavor and the development of certain of these compounds. In future quantitative work dealing with flavor of CS hams, the possibility should be taken into consideration that carbonyl compounds do make some contribution to the flavor complex.

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The Fatty Acid Composition of Liver Lipids from Rats Raised on Pork Rations[a, b]

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(Manuscript received July 3, 1963)

SUMMARY

The liver lipid fatty acid composition of animals raised on pork rations was determined and compared with that of animals raised on a stock ration. The pork rations contained approximately 25% crude lipid, the proportion of oleic acid being 46–50%. The relation between the dietary and liver lipid fatty acid compositions was evaluated, with the most striking relationship being that between dietary and liver lipid oleate/linoleate ratios. The tissue levels of oleic, linoleic, and arachidonic acids provided supporting evidence for the existence of a competitive effect of oleic acid in the conversion of linoleic to arachidonic acid. The results suggest that the dietary oleate/linoleate ratio is of importance in essential fatty acid nutrition in rations containing appreciable quantities of oleic acid. The sex variable, as it applies to the relation between dietary and liver lipid, was also evaluated.

INTRODUCTION

The effect of dietary fat on the blood and tissue lipids has been of interest for many years. In recent years such studies have received an added impetus from improvements in technique, namely alkali isomerization and gas chromatography. Recently, particular emphasis has been placed on the relation between dietary lipids and blood lipids, particularly in relation to serum cholesterol level and the incidence of atherosclerosis (Kinsell *et al.*, 1958).

It has also been observed that dietary lipids may influence the lipid composition of subcellular particles (Horwitt *et al.*, 1960; Marco *et al.*, 1960). In this regard it has been noted that diets deficient in essential fatty acids result in changes in the biochemical function and physical structure of liver mitochondria (Tulpule and Williams, 1955; Levin *et al.*, 1957).

Rats raised on rations containing irradiated beef or pork have shown increased liver cytochrome oxidase activity (Tinsley *et al.*, 1960). An increase in liver cytochrome oxidase activity has also been observed in rats raised on rations deficient in essential fatty acids (Tulpule and Williams, 1955). Therefore, recent experiments in this laboratory were designed to determine whether the increase in enzyme activity induced by the irradiated meat rations is the result of changes in the dietary lipid resulting from irradiation-induced oxidation. These experiments also afforded an opportunity to make some preliminary observations on the relation between dietary and liver lipids. The characteristic feature of the pork rations used is the high level of oleic acid.

EXPERIMENTAL

The composition of the pork rations is given in Table 1. The pork constituted 70% of the ration solids, and the crude lipid content of the ration was approximately 25%. The composition

Table 1. Composition of pork ration.

Components	g
Cooked pork meat	132
Salts	4
Guar gum	4
Potato starch	20.9
Choline chloride	0.3
Vitamin pre-mix	0.1
Liver extract	0.5
Inositol	0.2
Total solids	100

[a] Technical Paper No. 1482, Oregon Agricultural Experiment Station.

[b] This paper reports research undertaken in cooperation with the Office of the Surgeon General, Department of the Army, under contract DA-49-007-MD 580. The opinions expressed are those of the author and are not necessarily endorsed by the Department of the Army.

of the vitamin premix, the levels of fat-soluble vitamins, the description of ration components, and the methods of ration preparation and feeding were summarized by Buhl and Butts (1960).

Three different pork rations were used, and the prior treatment of the pork samples was as follows:

Pork I. Stored 12 months at −10°F before use

Pork II. Gamma-irradiated (5.6 megarad), stored 12 months at room temperature before use

Pork III. Fresh sample obtained prior to the experiment and held at −10°F for the duration of the experiment.

For comparison, a number of animals received the regular stock ration (A. E. Staley's Rockland rat diet), which contains 4% crude lipid.

This paper summarizes observations from two separate experiments. In the first experiment, two pork rations (I and II) and the stock ration were used. Eight male animals were weaned onto each of the rations and raised on these rations for 12 weeks before sacrifice and liver lipid analysis. In the second experiment, the three pork rations were used and eight animals (4 males and 4 females) were weaned onto each ration and again raised on the rations for 12 weeks before sacrifice and assay. The animals used were from our stock colony of Wistar-strain animals.

After sacrifice and exsanguination, the livers were excised and held in frozen storage for fatty acid analysis. A 2-g sample of the liver was taken, and the lipids were extracted with the chloroform-methanol procedure of Folch *et al.* (1957). A portion of the lipids obtained were saponified and then esterified with methanolic HCl. The procedures used were those outlined by James (1960). The fatty acid composition of the dietary lipids was obtained in a similar manner.

The fatty acid methyl esters were analyzed with a Beckman GC_2 gas chromatograph fitted with a hydrogen flame detector. A 6-ft × ⅛-inch O.D. column of 20% LAC-446 on firebrick was used at 190°C with helium as carrier gas.

RESULTS AND DISCUSSION

The pork rations were readily accepted by the rats and gave a growth rate comparable to that observed in animals raised on the stock rations. Extensive studies in this laboratory have indicated that the ingestion of pork irradiated to 5.6 megarad does not produce any gross physiological or histopathological changes (Buhl and Butts, 1960).

Relation between dietary and liver lipid fatty acid composition. The fatty acid composition of the ration lipids is given in Table 2. The stock ration lipids contained a high level of linoleate, with lower levels of oleate, stearate, and palmitate.

The fatty acid composition of the liver lipids of animals raised on these rations is summarized in Table 3. It is noted from the standard deviations that there is a considerable variation in liver lipid fatty acid composition among animals on the same ration.

No statistically significant differences in liver lipid fatty acid composition were obtained in animals raised on pork rations I and II. In experiment 1, the liver lipid fatty acid composition of animals raised on the stock rations differed from that of animals raised on the pork rations. In experiment 2, the pork ration III resulted in statistically significant changes in the level of oleate, linoleate, and arachidonate of the liver lipid when compared with pork rations I and II.

Fig. 1 illustrates the relation between fatty acid ratios of dietary and liver lipid as observed in these experiments. This figure gives the mean and the standard deviation for a set of observations. The difference in liver lipid ratios observed in the two experiments is due in part to the fact that both male and female animals were used in the second experiment, and only males in the first experiment.

Since it is known (Shorland, 1962) that, in liver tissue, palmitic and stearic acids are readily interconvertible, the ratio of these

Table 2. Fatty acid composition (%) of dietary lipid.

Ration	Palmitate	Palmitoleate	Stearate	Oleate	Linoleate	Linolenate	Stearate oleate	Oleate linoleate	Palmitate stearate
Stock	20.8	3.6	6.5	32.2	35.1	1.9	.202	0.92	3.20
Pork I	26.0	4.8	11.1	50.1	7.9	....	.222	6.35	2.34
Pork II	26.8	6.2	10.8	46.9	9.2	....	.230	5.10	2.48
Pork III	24.5	3.0	13.6	46.7	12.2	....	.291	3.83	1.80

Table 3. Fatty acid composition (%) of liver lipids.

Ration	Palmitate	Palmitoleate	Stearate	Oleate	Linoleate	Arachidonate	Stearate oleate	Oleate linoleate	Palmitate stearate
Experiment 1									
Stock	24.0±3.4	3.2±1.5	21.2±2.6	17.7±1.9	15.8±1.7	18.0±3.7	1.218	1.12	1.16
Pork I	21.7±1.7	1.5±0.5	17.4±3.0	37.4±4.8	8.3±3.3	13.8±3.3	.479	4.50	1.28
Pork II	20.9±1.1	1.4±0.7	18.1±3.6	35.4±6.1	9.6±1.1	14.6±4.0	.542	3.69	1.20
Experiment 2									
Pork I	♂ 21.6±1.4	1.3±0.4	17.2±1.7	35.2±1.7	9.6±0.7	15.0±2.1	.490	3.67	1.27
	♀ 17.2±2.8	1.2±0.5	22.2±5.4	28.0±2.5	9.4±2.1	21.7±5.8	.799	2.98	.79
Pork II	♂ 22.0±1.7	1.3±0.3	18.6±2.4	32.6±4.4	10.4±0.7	15.1±2.8	.586	3.12	1.21
	♀ 19.7±1.8	1.2±0.2	21.8±3.3	29.3±3.3	9.7±0.7	18.4±1.7	.762	3.02	.91
Pork III	♂ 19.5±1.4	1.2±0.4	20.2±2.6	24.4±4.4	12.8±1.8	21.8±3.8	.860	1.91	.98
	♀ 19.3±1.1	1.1±0.4	24.2±3.5	22.0±3.7	12.6±2.2	20.9±3.3	1.151	1.75	.81

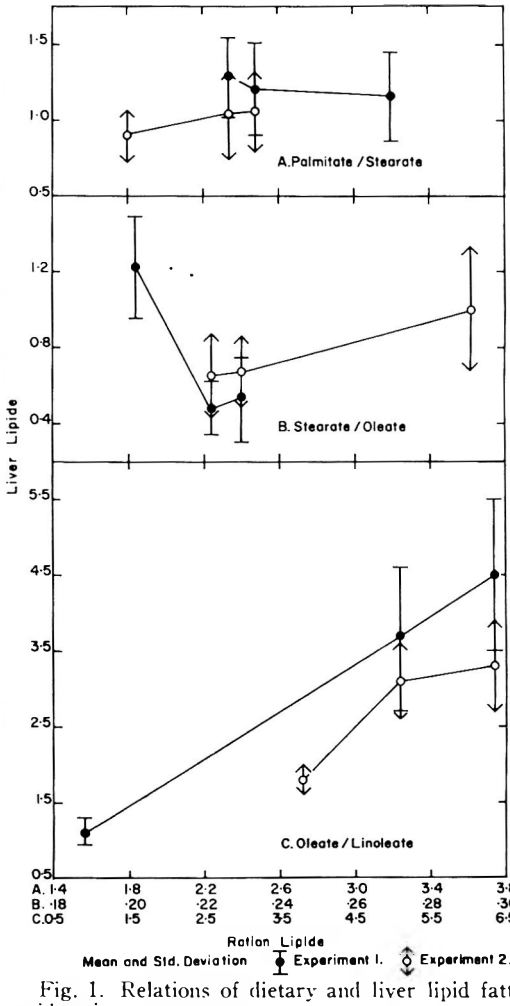

Fig. 1. Relations of dietary and liver lipid fatty acid rations.

acids should reflect the equilibrium maintained by the tissue. Should the dietary ratio be modified, it is possible that the tissue ratio would also be modified in the direction of the change in the dietary ratio if the metabolic processes were not adequate to cope with the change. Under the conditions of this experiment, liver tissue is able to maintain a constant palmitate-stearate ratio despite changes in the composition of the dietary lipid (Fig. 1-A). In both experiments the small differences in the tissue ratio were not statistically significant.

The interconversion of stearic and oleic acids has also been observed (Shorland, 1962), and hence the same reasoning could be applied to the tissue stearate-oleate ratio.

With pork rations, a direct relation between the dietary and tissue ratios is observed (Fig. 1-B), indicating the inability of the tissue to compensate for the dietary changes. The fact that the tissue ratio observed in the stock ration-fed animals does not conform to the relation observed in the pork animals is probably due to the difference in the lipid level of the stock and pork rations.

The following section describes a possible relation between arachidonic acid level and the oleate-linoleate ratio. Hence, the relation between the dietary and tissue oleate-linoleate ratio would be of interest. In Fig. 1-C it can be seen that the tissue ratio is dependent on the ratio of the ration. Despite the lower lipid content of the stock ration the tissue oleate-linoleate ratio conforms to the relationship observed in the pork-fed animals. Thus the inability of the tissue to compensate for such changes in the dietary lipid is of importance when the nutritional aspects of linoleic acid are considered.

Oleate-linoleate-arachidonate interaction. An inhibitory effect of oleic acid in essential fatty acid nutrition has been reported by Dhopeshwarkar and Mead (1957). It was suggested by these workers that oleic acid inhibited competitively the transformation of linoleic acid to arachidonic acid. Since the pork rations contained high levels of oleic acid the data obtained from analyses of liver lipids gave opportunity to study this effect.

The relation between the levels of oleic and arachidonic acids in liver lipids of rats raised on pork rations is given in Fig. 2. A highly significant correlation is obtained indicating that increasing amounts of oleic acid repress the formation of arachidonic acid. No such relation was observed in animals raised on the stock ration which had a lower lipid content and a considerably lower oleate-linoleate ratio than the pork rations (Table 2).

In the samples of liver lipids studied, the linoleate level varied from 6.8 to 15.6%, which variation should also influence the arachidonate level. If a competitive relation exists between linoleic and oleic acid one might expect a relation between the

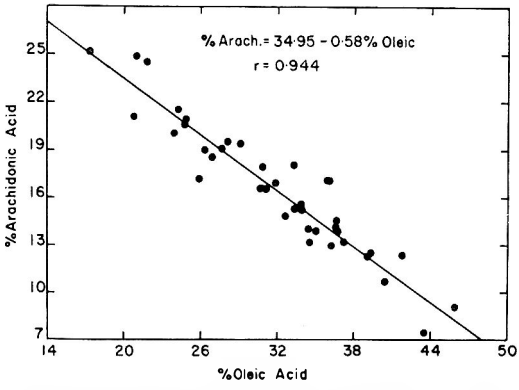

Fig. 2. Relation between proportions of oleic and arachidonic acids in liver lipid from animals raised on the three pork rations.

oleate-linoleate ratio and arachidonate level. A significant correlation is obtained between these quantities (Fig. 3).

The question arises as to whether the effect of oleic acid is due merely to dilution. In such a case the level of oleic acid would not influence the arachidonate-linoleate ratio in any regular fashion. This situation is evaluated in Fig. 4 and for limited ranges of the linoleate level, highly significant correlations are observed between these two quantities (Table 4). Thus the effect of oleic acid cannot be attributed merely to dilution. The regular manner in which the product-precursor ratio is influenced both by oleate and linoleate is most suggestive of a competitive interaction between these acids in the formation of arachidonic acid.

A direct relation is shown between the dietary and liver lipid oleate-linoleate ratio,

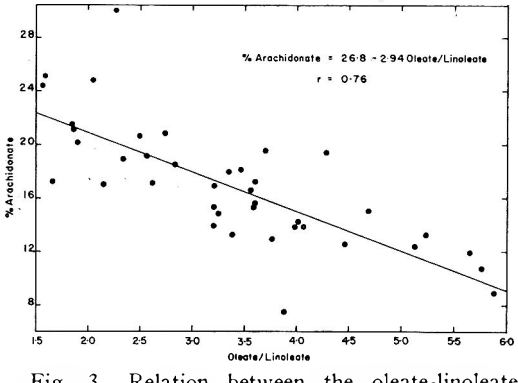

Fig. 3. Relation between the oleate-linoleate level and the level of arachidonic acid in liver lipids from animals raised on the three pork rations.

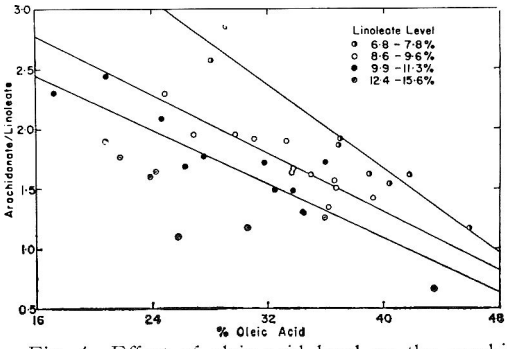

Fig. 4. Effect of oleic acid level on the arachidonate-linoleate ratio in liver lipids from animals raised on the three pork rations.

and it has also been demonstrated that the tissue arachidonate level is influenced by the oleate-linoleate ratio of the tissue. In view of these two facts, an important quantity to be considered in essential fatty acid nutrition in rations containing appreciable proportions of oleic acid would be the dietary oleate-linoleate ratio.

It should be noted that no symptoms of essential fatty acid deficiency were observed. The dietary oleate-linoleate ratio in the pork rations was in the range 3.8–6.4, which would be comparable to the ratio of approximately 5 expected in coconut oil, which was used in the control rations of Dhopeshwarkar and Mead (1961). Those workers observed slower growth of guinea pigs when fed a ration containing 3.65% coconut oil and 3.65% oleic acid or methyl oleate. In this case one might expect a dietary oleate-linoleate ratio approaching 60. Thus, a critical region for the dietary ratio must exist between 5 and 60, in which region gross deficiency symptoms would first be observed.

Holman (1960) used the tissue trienoic-tetraenoic acid ratio as an index of essential fatty acid nutrition. Increasing levels of tissue oleate would be expected to increase this ratio in two ways: 1) by increasing the proportion of 5,8,11-eicosatrienoic acid, this acid being derived from oleic acid (Dhopeshwarkar and Mead, 1961; Mead and Slaton, 1956); and 2) by suppressing the conversion of linoleate to arachidonate. It is suggested that the fatty acid triene-tetraene ratio would be determined in large part by the oleate-linoleate ratio in the tissue. More comprehensive studies are in progress to evaluate the hypotheses suggested by these results.

Sex variation in liver lipid fatty acid composition. The second experiment afforded an opportunity to evaluate the sex variable as related to the dietary liver lipid situation. A statistical analysis of the data is summarized in Table 4. It is evident that sex influences the fatty acid composition of the liver lipids. Female animals show decreased levels of palmitate and oleate and a decrease in the palmitate-stearate ratio. An increase in the proportion of stearate and in the stearate-oleate ratio was also observed in female animals. Sex did not influence the tissue level of linoleate.

The difference in level of arachidonic acid was not statistically significant over the whole experiment; this was probably due to a large variation in the observed levels of arachidonate. Table 3 shows that animals on the pork III ration showed no sex effect in the arachidonate level of the liver lipids. However, animals raised on the pork I and II rations showed a marked sex effect; levels of arachidonic acid were higher in females. Since there is no sex effect on liver linoleate level, the increase in arachidonate level must be associated with the decrease in level of oleic acid in the liver lipids of females. From Fig. 2, the levels of arachidonate corresponding to 30.7 and 26.6% oleic acid would be 17.2 and 19.6%, respectively, which values compare quite favorably to the observed values (Table 4).

Table 4. Summary of statistical analysis of data presented in Fig. 4.

Linoleate level	Regression equation	Correlation ratio
6.8– 7.8	arachidonate/linoleate=5.22—.0894 oleate	.974
8.6– 9.6	arachidonate/linoleate=3.74—.0606 oleate	.925
9.9–11.3	arachidonate/linoleate=3.36—.0574 oleate	.906

Table 5. Sex effect on liver lipid fatty acid composition (%).

Palmitate	Stearate	Oleate	Linoleate	Arachidonate	Palmitate stearate	Stearate oleate
♂ 21.0	18.7	30.7	11.0	17.3	1.152	.646
♀ 18.7	22.9	26.6	10.6	20.1	.836	.902
Sig. 0.5%	Sig. 1%	Sig. 1%	n.s.	n.s.	Sig. 5%	Sig. 2.5%

It has been reported that the essential fatty acid requirement of female rats is less than that of male rats (Anisfeld *et al.*, 1951; Greenberg *et al.*, 1950). From our data one would anticipate lower linoleate requirements in female rats than in male rats when the rations contained appreciable quantities of oleic acid or in rations in which the dietary oleate/linoleate ratio was high. Kushman and Coniglio (1961) observed no sex effects in the levels of polyunsaturated fatty acids in tissue up to six months of age. The rations used in those experiments contained 20% hydrogenated cottonseed oil, and one would not expect a high oleate/linoleate ratio in that situation.

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Water-Soluble Flavor and Odor Precursors of Meat. I. Qualitative Study of Certain Amino Acids, Carbohydrates, Non-Amino Acid Nitrogen Compounds, and Phosphoric Acid Esters of Beef, Pork, and Lamb[a]

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SUMMARY

Paper and ion-exchange chromatography were used to determine qualitatively certain constituents in lyophilized diffusates from cold-water extracts of beef, lamb, and pork muscles. The qualitative contents of low-molecular-weight diffusible organic constituents in tissue from these three species were remarkably similar. Involvement of constituents studied as flavor and odor precursors is discussed.

A popular aspect of food research is the investigation of chemical constituents involved in the production of desirable flavors and odors. Much attention has been devoted to fat-soluble constituents of meat (Pippen *et al.*, 1954, 1958; Henick *et al.*, 1954; Chang and Kummerow, 1955; Gaddis and Ellis, 1957, 1959; Berry and McKerrigan, 1958; Gaddis *et al.*, 1959, 1960; Hall *et al.*, 1962), but the water-extractable components have been studied sparingly in recent years. The amino acid and carbohydrate contents of meat are of extreme importance as potential flavor and odor precursors, and the products formed by the interaction of these materials during heating undoubtedly contribute to flavor and/or odor of cooked meat.

Several investigators have reported on the free organic constituents of meat (Greenwood *et al.*, 1951; Walker, 1952; Wood, 1956; Wood and Bender, 1957; Bender *et al.*, 1958; Ma *et al.*, 1961; Thompson *et al.*, 1961; Körmendy and Gantner, 1962) but there is a general lack of agreement on the exact constituents present in water-extracts of meat, and the concurrent study of free amino acids in meat from the three most popular species of red meat animals has not been reported. Even fewer studies have been made of the other non-protein-nitrogen-containing constituents and carbohydrates of meat.

Certain precursors of cooked beef flavor and odor are extractable with cold water (Hornstein *et al.*, 1960; Batzer *et al.*, 1960). Many of the cooked-beef flavor and odor precursors are low-molecular-weight compounds that diffuse through cellulose dialysis membranes. Dialysis techniques were first used in this type of study by Hornstein and Crowe (1960), and later by other investigators (Batzer *et al.*, 1960, 1962). The latter investigators used dialysis techniques to isolate a fraction from cold-water extracts of beef that contained glucose, inosinic acid, and an unknown glycoprotein. They indicated that all these compounds may be involved in the production of cooked-beef flavor and odor. The unknown glycoprotein contained glucose and several amino acids. It was suggested that, possibly, only certain of the amino acids present in the glycoprotein were necessary for the production of meat flavor. Wood (1956), Wood and Bender (1957), Bender *et al.* (1958), and Wood (1961) presented a series of excellent reports on the use of paper chromatography for analyzing certain ox-muscle extracts. It has been demonstrated that various combinations of amino

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acids and carbohydrates produce cooked-meat-type flavors and odors upon heating (May, 1960) and that individual amino acids when heated with glucose at different temperatures produce a large number of different aromas (Herz and Shallenberger, 1960). A simultaneous non-enzymatic browning reaction was observed in the amino acid–carbohydrate reaction mixtures. Literature on amino acid–carbohydrate interactions was reviewed extensively by Danehy and Pigman (1951) and Hodge (1953).

This study was made to determine qualitatively some of the non-polymeric water-soluble nitrogen-containing substances and carbohydrates of fresh beef, lamb, and pork muscle.

EXPERIMENTAL

Preparation of lyophilized diffusates used in this study. The samples were prepared by overnight extraction of lean U. S. Choice beef round, leg of lamb, and lean fresh ham muscle with equal weights of cold distilled water with occasional stirring. A few drops of chloroform were added to each extraction mixture to inhibit bacterial growth, and the temperature was maintained at 2°C. The slurries were drained through cheesecloth for 2 hr and the cheesecloth squeezed by hand to remove as much of the extract from the residue as possible. Three-hundred ml of each extract was dialyzed 24 hr at 2°C against 5 volumes of distilled water. The dialysates were lyophilized to produce white, fluffy powders, which were very hygroscopic and upon standing at room temperature turned brown and developed a meaty odor. Three hundred mg of each lyophilate was dissolved in 6 ml of distilled water and desalted in an electric desalter (RSC, Model 1930). The samples were desalted at 40 volts until the amperage decreased to 0.2, and then diluted to 10 ml with distilled water. The samples were kept at −25°C until used for the following experiments:

Multi-solvent systems and paper chromatography. Twenty microliters of each desalted sample was spotted at 2-cm intervals along a line drawn 2 cm from the bottom of a sheet of Whatman No. 1 filter paper (25 × 30 cm) along with 5-μl portions of the individual and mixed amino acid standards. The standards were spotted on the left side of the papers. The filter papers were stapled into circles and developed according to the procedures described by Block and Weiss (1956) for amino acids in groups A, B, and C. The papers were dipped in 0.25% ninhydrin in acetone and placed in a darkened hood overnight for color development. The amino acids in the unknown samples were identified by comparing their migration rates and colors and shapes of the spots with those of the amino acid standards.

Two-dimensional paper chromatography with phenol and pyridine. Fifty-microliter portions of the desalted beef, lamb, and pork samples were applied 6.5 cm from one edge and 6.5 cm from the bottom of separate sheets of Whatman No. 1 filter paper (22 × 22 in). One paper was prepared with a 10-μl portion of a solution containing 20 standard amino acids. The papers were developed in a chromatocab (Reco No. 8145) according to the procedure of Consden *et al.* (1944), using phenol-water (100:20 v/v) in one dimension and pyridine-water (80:20 v/v) in the other dimension. Color development was as described above.

To determine the migration rates of the individual amino acids, chromatograms of standard amino acids were prepared in the solvent systems used, and these were plotted on a two-dimensional map. This was compared with the two-dimensional chromatograms of the beef, lamb, and pork samples and the ninhydrin-positive spots identified.

Two-dimensional paper chromatography with phenol and collidine. The development technique described previously was used, except that 75 μl each of the beef, lamb, and pork samples were analyzed and the solvent for development in the second dimension was collidine-water (125:44 w/v). It was found that colors characteristic of each amino acid were developed after the papers were sprayed with 0.25% (w/v) ninhydrin in acetone. Identification of the ninhydrin-positive spots on the two-dimensional chromatograms was as previously described.

Ion-exchange chromatography to identify amino compounds in diffusates. The ion-exchange method of Moore *et al.* (1958), revised as described below, was used to check the results of the paper chromatography. Sulfonated polystyrene cation-exchange resin (Aminex MS cation-exchange resin, fractions C and D, Bio-Rad Laboratories) columns were used to separate the amino compounds, and the effluent was analyzed by the ninhydrin method of Rosen (1957) as modified by Wood (1959). The amino compounds in the effluent were identified by comparison of the elution volumes of the unknowns with those of authentic compounds eluted under similar conditions. Samples were prepared by dissolving 1 g each of the lyophilized diffusate powder from beef, lamb, and pork in 5 ml of distilled water. Half of each solution was diluted to 10 ml, and 2-ml portions were diluted to 8 ml with pH 2.2 sodium citrate buffer. Two-ml portions containing 25 mg of the diffusate powder were used on the column for

determining the acidic and neutral amino compounds, and 1-ml portions containing 12.5 mg of the diffusate powder were used for determining the basic amino compounds.

One-dimensional descending paper chromatography to determine carbohydrates. One gram of lyophilized diffusate powder from each species was dissolved in 2.5 ml of distilled water, and 1.25-ml portions were diluted to 10 ml. Five-ml portions were desalted as described previously and diluted to 10 ml. Fifty microliters of each unknown, and 5-μl portions of carbohydrate standards containing ribose, glucose, fructose, and maltose were spotted 2 cm apart on a line drawn 6.5 cm from the edge of a sheet of Whatman No. 1 filter paper. The carbohydrate standards contained 0.25, 0.50, 0.75, and 1.0 micromoles of each sugar in 5 μl. The solvent system was *n*-butanol–acetic acid–water (5:1:4 v/v; Hirst and Jones, 1949), and sugars were identified by migration rates and colors after reaction with aniline hydrogen oxalate (Partridge, 1949).

Qualitative analyses of non-amino acid nitrogen compounds and phosphoric acid esters. One-dimensional ascending paper chromatography was used with the solvent system and filter paper described for carbohydrate determinations. The following standard materials were prepared to contain 0.1 μg phosphorus per μl: inosine monophosphate, ribose-5-phosphate, fructose-1,6-diphosphate, fructose-6-phosphate, adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, phosphoserine, phosphoethanolamine, glucose-1-phosphate, glucose-6-phosphate, and diphosphopyridine nucleotide. Standards containing 2.0 μg per μl of hypoxanthine, inosine, creatine, and creatinine were also prepared. Fifteen-microliter portions of each standard were spotted at 2-cm intervals along a line drawn 2 cm from the bottom of each paper. Fifty microliters of the beef, lamb, and pork samples were examined along with the standards. Spots on the chromatograms were located by irradiation with ultraviolet light (Bandurski and Axelrod, 1951) or by spraying with the molybdic acid reagent of Hanes and Isherwood (1949). After spraying, the chromatograms were air-dried in a hood, then heated 5 min in an oven at 85°C to develop colors. The phosphorus-containing spots were dark-blue against a light-blue background. Spots detected were identified by comparing their R_f values with those of standards.

RESULTS AND DISCUSSION

Multisolvent systems and paper chromatography for identifying amino acids. The R_f values obtained from development of standard amino acids for groups A, B, and C agree closely with those reported by Block and Weiss (1956). Comparisons of R_f, R_a, and R_v values of the ninhydrin-positive spots of the unknowns with those of the standards indicated that alanine, arginine, aspartic acid, cystine, glutamic acid, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine were present in samples from beef, lamb, and pork. The lamb sample produced at R_v 1.16 a spot that was absent in the beef and pork samples, and the beef and pork samples had respective spots at R_v 0.63 and 0.66 that were absent in the lamb sample.

Two-dimensional paper chromatography with phenol and pyridine. Amino components identified by this method in diffusate concentrates from beef, lamb, and pork, presented in Table 1, included alanine, aspartic acid, cysteine, glutamic acid, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, valine, carnosine, 1-methylhistidine, phosphoserine, and taurine. In addition, hydroxyproline was identified in samples from beef and lamb, and cysteic acid and glutathionine were identified in samples from lamb.

Two-dimensional paper chromatography with phenol and collidine. Table 1 lists amino compounds identified in diffusate concentrates of beef, pork, and lamb using paper chromatography developed with phenol and collidine. Thirteen ninhydrin-positive spots were developed on the chromatogram of the beef sample. Separation of the components appeared to be complete except for isoleucine, leucine, and methionine, and the spots were small and well-defined. The colors and R_f values in both solvent systems corresponded to those of the standard compounds. Eleven spots were detected on the chromatogram of pork. Isoleucine, leucine, methionine, and phenylalanine were not separated. The chromatogram of lamb contained 12 ninhydrin-reactive substances.

Ion-exchange chromatography to identify amino compounds. Separation of the amino compounds by the ion-exchange method was excellent, although in some cases the peaks overlapped. The proline peak was partially masked by glutamic acid and citrulline peaks in the standards. In the unknowns, citrulline was not detected and

Table 1. Amino compounds identified in tissue of beef, pork, and lamb by paper and ion-exchange chromatography.

Amino compound identified	Method of identification											
	Beef				Pork				Lamb			
Alanine	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
Arginine	(1)			(4)	(1)			(4)	(1)			(4)
Aspartic acid	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
Cysteine		(2)				(2)				(2)		
Cystine	(1)			(4)	(1)			(4)	(1)			(4)
Glutamic acid	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
Glycine		(2)	(3)	(4)		(2)		(4)		(2)	(3)	(4)
Histidine				(4)				(4)				(4)
Hydroxyproline		(2)					(3)			(2)		
Isoleucine		(2)[a]	(3)[a]	(4)		(2)[a]	(3)[a]	(4)	(1)	(2)[a]	(3)[a]	(4)
Leucine	(1)	(2)[a]	(3)[a]	(4)	(1)	(2)[a]	(3)[a]	(4)	(1)	(2)[a]	(3)[a]	(4)
Lysine	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
Methionine	(1)	(2)	(3)[a]	(4)	(1)	(2)	(3)[a]	(4)	(1)	(2)	(3)[a]	(4)
Phenylalanine	(1)[a]	(2)	(3)[a]	(4)	(1)[a]	(2)	(3)[a]	(4)	(1)[a]	(2)	(3)[a]	(4)
Proline	(1)		(3)	(4)	(1)		(3)	(4)	(1)		(3)	(4)
Serine	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
Threonine	(1)	(2)	(3)	(4)	(1)	(2)		(4)	(1)	(2)	(3)	(4)
Tyrosine	(1)		(3)	(4)	(1)			(4)	(1)		(3)	(4)
Valine	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)	(1)	(2)	(3)	(4)
Anserine				(4)[c]				(4)[c]				(4)[c]
Carnosine		(2)		(4)[b]		(2)		(4)[b]		(2)		(4)[b]
Cysteic acid							(3)			(2)		
Glutathione										(2)		
Glutamine			(3)				(3)				(3)	
Glycerophospho-ethanolamine				(4)				(4)				(4)
1-Methylhistidine		(2)		(4)		(2)		(4)		(2)		(4)
Ornithine								(4)				(4)
Phosphoethanolamine				(4)			(3)	(4)				(4)
Phosphoserine		(2)	(3)	(4)		(2)	(3)	(4)		(2)	(3)	(4)
Taurine		(2)	(3)	(4)		(2)	(3)	(4)		(2)	(3)	(4)
Urea				(4)				(4)				(4)

(1) Multi-solvent paper chromatography (Block and Weiss, 1956).
(2) Two-dimensional paper chromatography: phenol and pyridine.
(3) Two-dimensional paper chromatography: phenol and collidine.
(4) Ion-exchange chromatography (Moore *et al.*, 1958).
[a] Compound possibly present, but not separated under conditions used.
[b] Not distinguished from carnosine.
[c] Not distinguished from anserine.

glutamic acid was present in sufficient quantity to partially obscure the proline peak. Serine and asparagine were eluted at approximately the same volume, so it was not possible to distinguish between them. The same was true of ammonia and lysine, and of anserine and carnosine. Resolution was good in most other areas on the chromatograms.

Amino compounds identified in the samples examined by ion-exchange are summarized in Table 1. The results obtained substantiated identification of alanine, aspartic acid, glutamic acid, serine, threonine, lysine, methionine, valine, phosphoserine, and taurine, which were identified in beef, lamb, and pork by at least two, and in most cases three, types of paper chromatography. The presence of glycine, proline, tyrosine, leucine, phenylalanine, cystine, arginine, and 1-methylhistidine in all three samples was indicated by at least one type of paper chromatography and by ion-exchange chromatography.

Cystine, histidine, hydroxyproline, isoleucine, carnosine, glutamine, glycerophos-

phoethanolamine, phosphoethanolamine, and urea were identified in samples from all three species by at least one type of chromatography. In addition, glutathione was identified in lamb, and cysteic acid and ornithine were identified in pork and lamb. Anserine was possibly present in samples from all three species, but this compound was not specifically identified by the method used.

These results indicate that the free amino acid contents of muscle from beef, pork, and lamb are qualititatively quite similar and in many respects are similar to those of fish (Shewan, 1955; Jones, 1959) and cat muscle (Tallan *et al.*, 1954).

One-dimensional descending paper chromatography to identify carbohydrates. Ribose, fructose, glucose, and an unknown compound that reacted with aniline hydrogen oxalate were present in beef, lamb, and pork. The unknown compound was tentatively identified as maltose, from its rate of migration, but the color developed with the aniline hydrogen oxalate more closely resembled that of ribose. The unknown compound was possibly a ribose derivative of a nucleic acid residue, perhaps inosine.

Qualitative analyses of non-amino acid nitrogen compounds and phosphoric acid esters. Inosine, creatinine, and hypoxanthine were identified in all samples of beef, lamb, and pork examined. Comparison of the sizes and intensities of spots on chromatograms of samples from all three species with known concentrations of the nitrogen-ring compound indicated that quantities of these compounds were relatively high in samples of all three types.

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Water-Soluble Flavor and Odor Precursors of Meat. II. Effects of Heating on Amino Nitrogen Constituents and Carbohydrates in Lyophilized Diffusates from Aqueous Extracts of Beef, Pork, and Lamb[a]

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SUMMARY

Quantitative analyses were made of water-extractable amino nitrogen and carbohydrate constituents of beef, lamb, and pork before and after heating. In all three species, taurine, anserine-carnosine, and alanine were present in relatively large quantities, and losses of these were large during heating. Other important amino acids degraded during heating were: glutamic acid, glycine, lysine, serine, cystine, methionine, leucine, isoleucine, and methyl histidine. Heating caused marked increases of phosphoethanolamine in samples from the three animal species studied. Ribose was the carbohydrate most labile to heating, and glucose was the most stable. The importance of these constituents as odor and/or flavor precursors of meat is discussed.

One of the most important aspects in the production of desirable flavors and odors in meat is the interaction of carbohydrate and amino nitrogen during heating to produce browned products. The chemical nature of these reactions is quite complicated as indicated in reviews by Jones (1959), Hodge (1953), and Ellis (1959), and exact identification of the end products involved is difficult. Another approach to the problem is to study the disappearance of certain constituents and relate this indirectly to browning and to the formation of desirable meat flavors and odors.

Since the precursors of desirable flavor and odor compounds in meat are largely water-soluble (Wood, 1956; Kramlich and Pearson, 1958; Hornstein *et al.*, 1960; Batzer *et al.*, 1960) and of relatively low molecular weight (Hornstein *et al.*, 1960; Batzer *et al.*, 1960), the constituents of diffusates from water-soluble extracts are of considerable importance.

Wood and Bender (1957), Wood (1956, 1961), and Bender *et al.* (1958) in a series of classic articles studied the relative contents of various nitrogen constituents and carbohydrates in water extracts of fresh ox muscle and in a commercial ox muscle extract produced by heating. The major changes due to heating involved losses in amino acids, phosphoric acid esters, glucose, and ribose.

In contrast to other studies, Tarr (1954) indicated that only free ribose was involved in Maillard reactions of fish muscle. He reported that beef and veal muscle form free ribose from ribosides and ribose-5-phosphate, whereas pork muscle formed free ribose from ribose-5-phosphate.

The importance of the interaction between amino nitrogen compounds and carbohydrates in the production of odors was reported by Herz and Shallenberger (1960), who studied reactions of these compounds at relatively high temperatures, and by Keeney and Day (1957), who discussed the importance of the Strecker degradation of amino acids in flavor development in cheese.

This study was made to determine quantitatively the effects of heating on water-soluble amino compounds and carbohydrates from fresh beef, lamb, and pork muscle.

EXPERIMENTAL

The effect of heating was determined on some constituents of the lyophilized diffusates from water extracts of beef, lamb, and pork described

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previously (Macy *et al.*, 1964). Quantitative analyses were made of the individual amino compounds, individual carbohydrates, and total carbohydrates before and after heating.

The procedures used to prepare samples prior to analyses of the above constituents were as follows: Samples used for the analysis of individual amino acids and sugars were prepared by dissolving 1 g of each dried powder in 5 ml distilled water. Half of each solution, containing 200 mg/ml, was pipetted into 10-ml volumetric flasks and heated 1 hr in a boiling-water bath. This resulted in extensive browning and typical "brothy" odors in all cases. The heated and unheated samples were diluted to 10 ml with distilled water prior to chromatographic analysis. A similar procedure was used for analyses of total carbohydrate before and after heating, except that 200 mg of the dried lyophilisates were heated in 0.5 ml water, and the final dilution was to 4 ml.

Quantitative determination of individual amino acids. For amino acid analysis, 2-ml portions of the above samples were diluted to 8 ml with pH 2.2 sodium citrate buffer, and 2-ml portions were used for determining the basic amino compounds. Concentrations of amino compounds were calculated as mg per 100 g wet tissue.

The ion-exchange method of Moore *et al.* (1958) was used. The amino acids were separated on sulfonated polystyrene cation-exchange resin columns and determined spectrophotometrically. The resins (Aminex MS cation-exchange resin, fractions "C" and "D," Bio Rad Laboratories) were used without further purification. Two 150-cm columns were prepared to separate acidic and neutral amino acids, and one 50-cm column was prepared to separate basic amino acids. A flow rate of 18 ml per hour was maintained through the 150-cm columns by 7.5 lb/in.^{2} air pressure above the buffer reservoirs. The flow rate of the 50-cm column was 12.5 ml per hour at 9 lb/in.^{2} air pressure.

A calibration mixture of standard amino acids (Spinco Division Beckman Instruments, Inc., Model 120 calibration mixture) was used to calibrate the 150-cm columns. It contained lysine, histidine, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, tyrosine, phenylalanine, and ammonium chloride. In addition, the following amino compounds were prepared as a calibration mixture and determined separately: phosphoserine, phosphoethanolamine, taurine, asparagine, citrulline, and β-alanine. The standard-mixture stock solutions were at concentrations of 2.5 micromoles per ml in hydrochloric acid solution (1 ml concentrated HCl per 100 ml solution). Two-ml portions of the stock solutions were diluted to 10 ml with pH 2.2 sodium citrate buffer prior to use on the columns. The Spinco calibration mixture was used as the source of lysine, histidine, arginine, and ammonium chloride for calibration of the 50-cm column. In addition, a calibration mixture containing carnosine, anserine nitrate, 1-methylhistidine, ornithine, and γ-amino-n-butyric acid was prepared as described previously. Two-ml portions of the stock solutions were diluted to 10 ml with pH 2.2 sodium citrate buffer. Four ml of each of the two solutions were mixed, resulting in a concentration of 0.25 micromoles of each amino compound per ml. Two-ml portions containing 0.5 micromoles of each amino compound were used to calibrate the 50-cm column.

The method of Rosen (1957) as modified by Wood (1959) was used to analyze the effluent fractions. The absorbance values were plotted on K & E cross-section millimeter graph paper versus ml effluent so that 20 mm on the y-axis equalled 0.1 absorbance unit and 1 mm on the x-axis equalled 1 ml effluent. The areas under the peaks were integrated by a modification of the method of Spackman *et al.* (1958) for the integration of areas under peaks obtained from an automatic amino acid analyzer. Half the maximum absorbance, corrected for baseline absorbance, was multiplied by the estimated width of the peak at the base. This was the integration value for each peak on the chromatograms. The integration values of the standard amino acids, divided into the integration values of the unknown amino acids and multiplied by the concentrations (micromoles) of standard amino acids, were the concentrations of the unknown acids.

Quantitative determination of individual carbohydrates. Five-ml portions of the samples described for amino acid analysis were desalted in an electronic desalter (RSC, 1930) at 40 V until the amperage dropped to 0.2. The desalted samples were diluted to 10 ml with distilled water, resulting in a final concentration of 25 mg/ml of each of the respective diffusate powders. The paper chromatographic method of Bailey (1953) was modified for determination of the individual carbohydrates present in the samples. The concentrations of the developed spots on the papers were determined, with a Photovolt densitometer to measure the maximum absorbance of each spot, and a K and E compensating polar planimeter to determine the spot areas. The maximum absorbance multiplied by the total spot area was related to concentration.

Carbohydrate standards used contained 0.625, 1.25, and 2.5 micromoles of glucose, ribose, and maltose in 5 μl. Five-μl portions were spotted on the papers with the unknowns, and separated concurrently. n-Butanol–acetic acid–water (4:1:5

v/v), described by Partridge (1948), was used for chromatographic development. The papers were developed 40 hr by the descending technique. They were air-dried and dipped in aniline hydrogen oxalate (Partridge, 1950), dried, and heated 5 min at 100°C. The chromatograms were cut into 2-cm strips, and absorbance was measured with the densitometer. Outlines of the spots were drawn, and their areas were determined with a planimeter.

Quantitative determination of total carbohydrates. The anthrone method of Loewus (1952) was used to determine total carbohydrate contents of the above samples. Results are reported as mean values obtained from analyses of duplicate samples at two dilutions. Standard glucose samples at concentrations from 5 to 80 μg were analyzed.

RESULTS AND DISCUSSION

Quantitative analysis of amino acids and similar compounds. The precision of the method used for determining individual amino compounds is indicated in Table 1. The results are integration values obtained from analysis of four samples of standard (micromole) amino compounds, except samples marked with superior letters. Those results are within the precision expected for this type of analysis. In most instances the percent relative standard deviation was less than 4.0.

Quantities of individual amino compounds determined in heated and unheated lyophilized diffusate samples from beef, lamb and pork are presented in Tables 2, 3, and 4.

The amino nitrogen compounds of samples from the three species appear to be quite similar quantitatively, as they were qualitatively (Macy *et al.*, 1964).

In all three cases, taurine, anserine-carnosine, and alanine were the major constituents of unheated samples, and losses during heating were relatively large. Decreases in these constituents accounted for 69, 72 and 45% of the total loss during heating of this type of compound in beef, pork, and lamb, respectively. Jones (1959) also found that taurine was one of the major contributors to browning in fresh and muscle extracts. Other important constituents degraded during heating were glutamic acid, glycine, lysine, serine, cystine, methionine, leucine, isoleucine, and methyl histidine. The loss in essential amino acids ranged from approximately 20% in pork samples to approximately 55% in beef samples.

Table 1. Mean integration values and standard deviations of standard amino compounds determined by ion-exchange chromatography.[a]

Compound	Integration value	Relative standard deviation (%)
Aspartic acid	2.36±0.0410	1.7
Threonine	2.40±0.0732	3.1
Serine	2.35±0.0511	2.2
Glutamic acid	2.11±0.0115	0.5
Proline	0.53±0.0166	3.2
Glycine	2.10±0.0509	2.4
Alanine	2.13±0.0265	1.2
Cystine	1.62±0.0370	2.3
Valine	2.33±0.0863	3.7
Methionine	1.72±0.0726	4.2
Isoleucine	1.88±0.0673	3.6
Leucine	1.98±0.0703	3.6
Tyrosine	2.17±0.0291	1.3
Phenylalanine	2.33±0.0208	0.9
Phosphoserine [b]	3.22±0.0826	2.6
Phospho-ethanolamine [b]	0.95±0.0187	2.0
Taurine [b]	2.29±0.0587	2.6
Asparagine [b]	1.77±0.0483	2.7
Citrulline [b]	2.36±0.0628	2.7
β-alanine [b]	2.15±0.0330	1.5
Ornithine [c]	2.53±0.0720	4.1
γ-amino-*n*-butyric acid [c]	2.19±0.0030	0.2
1-methylhistidine [b]	2.62±0.0230	1.3
Ammonia + lysine	5.66±0.0090	0.2
Histidine [c]	2.14±0.0390	2.6
Anserine-nitrate [c]	1.39±0.0180	1.8
Carnosine [c]	1.57±0.0280	2.5

[a] Moore *et al.* (1958).
[b] Mean of three determinations.
[c] Mean of two determinations.

The degradation of some of these products obviously accounts for the appearance of hydrogen sulfide, methylmercaptan, acetaldehyde, propionaldehyde, and other substances (Bender and Ballance, 1961) produced oxidatively during the heating of meat and meat extracts.

The phosphoethanolamine contents of samples from all three species increased, and phosphoserine and glycerophosphoeth-

Table 2. Quantities of amino compounds in heated and unheated lyophilized diffusate powder from beef determined by ion-exchange chromatography.[a]

Amino compound	mg/100 g tissue		Change from unheated sample (%)
	Unheated	Heated	
Phosphoserine	0.36	0.29	— 19.4
Glycerophosphoethanolamine	0.02	0.01	— 50.0
Phosphoethanolamine	0.66	0.82	+ 24.2
Taurine	9.05	4.02	— 55.6
Urea	0.01	....	—100.0
Aspartic acid	0.82	0.32	— 61.0
Threonine	1.11	0.74	— 33.3
Serine + asparagine (as serine)	7.53	1.35	— 82.1
Glutamic acid	4.63	2.22	— 52.1
Glycine	2.40	1.32	— 45.0
Alanine	11.28	6.22	— 44.9
Cystine	4.37	....	—100.0
Valine	2.99	1.47	— 50.8
Methionine	2.01	0.75	— 62.7
Isoleucine	2.04	0.87	— 57.4
Leucine	3.81	2.34	— 38.6
Tyrosine	1.85	0.80	— 56.8
Phenylalanine	1.36	0.97	— 28.7
NH_3 + lysine	6.19	4.11	— 33.6
Histidine	4.10	4.17	+ 1.7
Anserine + carnosine (as carnosine)	90.14	38.17	— 57.7
1-methylhistidine	4.80	0.49	— 89.8
Total	161.53	71.45	— 55.77

[a] Moore *et al.* (1958).

anolamine in pork decreased during heating. This indicates hydrolysis of phospholipids or similar compounds during heating of meat. Other constituents increasing in quantity during heating were histidine and methionine. The increase in histidine was probably due to hydrolysis of carnosine and/or anserine.

Considering the total amounts of amino compounds determined by these methods, beef contained the largest amount of these compounds, and pork the least. The percentages of the total amino compounds lost by heating were proportional to the initial quantities present. Greater quantities were lost from the beef samples than from the lamb or pork samples. Carbohydrates present in these samples were similarly affected by heating.

Quantitative analysis of carbohydrates. As pointed out in an earlier communication (Macy *et al.*, 1964), glucose, fructose, ribose, and some unidentified carbohydrates containing material that behaved similar to a nucleoside chromatographically, were found in extracts from beef, lamb, and pork. The contents of these sugars in samples from the three species were quite similar (Table 5); glucose was present in greatest quantities, followed by fructose, ribose, and the unknown fraction. Ribose appeared to be the most labile to heating, and fructose the most stable. Tarr (1954) reported that, in fish muscle, ribose liberated enzymically from ribonucleic acid was responsible for browning, and demonstrated that beef muscle could form ribose from added inosine. This strongly implicates inosine in reactions concerned with the browning of meat, and possibly its importance as a flavor

Table 3. Quantities of amino compounds in heated lyophilized diffusate powder from lamb determined by ion-exchange chromatography.[a]

Amino compound	mg/100 g tissue		Change from unheated sample (%)
	Unheated	Heated	
Phosphoserine	0.30	0.22	— 26.7
Glycerophosphoethanolamine	0.01	.....	—100.0
Phosphoethanolamine	1.15	1.90	+ 65.2
Taurine	26.25	16.47	— 37.3
Urea	trace	.....	.....
Aspartic acid	1.52	1.36	— 10.5
Threonine	3.14	1.36	— 56.7
Serine + asparagine (as serine)	4.74	2.26	— 52.3
Glutamic acid	6.08	2.85	— 53.1
Proline	3.05	0.83	— 72.8
Glycine	4.30	2.25	— 47.6
Alanine	9.18	6.53	— 28.9
Cystine	6.46	5.24	— 18.9
Valine	0.44	0.18	— 59.1
Methionine	2.37	1.53	— 35.4
Isoleucine	2.79	1.87	— 33.0
Leucine	5.62	3.06	— 45.6
Tyrosine	1.98	1.67	— 15.7
Phenylalanine	1.85	1.12	— 39.5
Ornithine	0.95	0.65	— 31.6
NH_3 + lysine	10.51	5.59	— 46.8
Histidine	9.70	7.16	— 26.2
Anserine + carnosine	25.55	16.28	— 36.3
1-methylhistidine	2.07	1.08	— 47.8
γ-amino-*n*-butyric acid	trace	.....	.....
Total	130.01	81.46	— 37.3

[a] Moore *et al.* (1958).

and/or odor precursor in these products.

Data in Tables 5 and 6 on total carbohydrate contents of beef, lamb, and pork indicate the importance of sugar concentration in the production of browned products, and consequently flavor production during heating. The percentage loss of carbohydrate is reasonably parallel to total sugar content.

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Table 4. Quantities of amino compounds in heated lyophilized diffusate powder from pork determined by ion-exchange chromatography.[a]

Amino compound	mg/100 g tissue		Change from unheated sample (%)
	Unheated	Heated	
Phosphoserine	0.35	0.23	— 34.3
Glycerophosphoethanolamine	0.02	0.01	— 50.0
Phosphoethanolamine	0.45	1.89	+320.0
Taurine	12.58	7.94	— 36.9
Aspartic acid	1.37	0.45	— 67.2
Threonine	0.48	0.45	— 6.3
Serine + asparagine (as serine)	2.95	0.53	— 82.0
Glutamic acid	1.95	1.19	— 39.0
Proline	0.64	0.24	— 62.5
Glycine	2.75	1.58	— 42.5
Alanine	4.19	2.80	— 33.2
Cystine	2.11	0.89	— 57.8
Valine	0.30	0.20	— 33.3
Methionine	0.69	1.00	+ 44.9
Isoleucine	1.03	0.83	— 19.4
Leucine	1.68	1.00	— 40.5
Tyrosine	0.56	0.37	— 33.9
Phenylalanine	0.51	0.39	— 23.5
Ornithine	trace	0.56	.....
NH_3 + Lysine	4.27	4.06	— 4.9
Histidine	2.55	3.11	+ 22.0
Anserine + carnosine (as carnosine)	67.94	58.38	— 14.1
1-methylhistidine	0.49	trace	—100.0
Total	109.86	88.10	— 19.8

[a] Moore *et al.* (1958).

Table 5. Concentration of various carbohydrates in lyophilized diffusates from beef, lamb, and pork before and after heating.

Carbohydrate	Concentration mg/100 g tissue					
	Beef		Lamb		Pork	
	Unheated	Heated	Unheated	Heated	Unheated	Heated
Glucose	43.86	25.25	32.87	21.33	43.56	28.02
% loss		42.4		35.1		35.7
Fructose	3.56	3.21	2.68	2.61	2.08	2.02
% loss		9.8		2.6		2.9
Ribose	1.09	trace	0.52	trace	0.20	trace
% loss		100		100		100
Unknown nucleoside (as ribose)	0.15	none	0.35	none	0.06	none
% loss		100		100		100
Total	48.66	28.46	36.42	23.94	45.90	30.04
% loss		41.5		34.3		34.6

Table 6. Effect of heating concentrated solutions of lyophilized diffusates from beef, lamb, and pork on carbohydrate content.

Type of sample	Total carbohydrate (mg/100 g tissue)		
	Unheated	Heated	% decrease
Beef	47.10	12.90	72.6
Lamb	23.92	10.66	55.4
Pork	43.35	15.30	64.7

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Some Volatile Components of Cooked Rutabaga[a]

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SUMMARY

Acetaldehyde, hydrogen sulfide, dimethyl sulfide, methyl disulfide and ammonia were identified in the volatiles of freshly cooked rutabaga; mercaptan(s) and isothiocyanates were detected. Some quantitative data are reported. Two procedures were devised for isolation of the volatile components of rutabaga for gas chromatographic examination. Typical chromatograms are included, with some peaks tentatively identified.

Some volatile chemical compounds, even in minute quantities, contribute significantly to the characteristic flavor of fresh or processed foods. In recent years a number of investigations have been carried out for the isolation, characterization, and identification of flavor components of different foods. Symposia on flavor research and chemistry have been reported (Mitchell *et al.*, 1957; Little, 1958; Campbell Soup Company, 1961; U. S. D. A., 1963). Relatively little investigation has been made of the flavor components of vegetables.

The literature on the chemistry of fruit and vegetable flavors up to 1948 was reviewed by Kirchner (1949). More recent are investigations of the components of a number of vegetables such as cabbage (Dateo *et al.*, 1957; Clapp *et al.*, 1959; Bailey *et al.*, 1961), onions (Niegisch and Stahl, 1956; Carson and Wong, 1961), peas (Ralls, 1960; Silberstein, 1954; Matthews, 1960), tomatoes (Spencer and Stanley, 1954; Matthews, 1960), snap beans (Matthews, 1960), string beans (Mackay *et al.*, 1958), cucumbers (Forss *et al.*, 1962), potatoes (Self *et al.*, 1963), and celery (Gold and Wilson III, 1961, 1963).

Rutabaga (*Brassica napus* var. *napobrassica*) is a root crop with a strong and characteristic flavor. "Bitterness," a poorly defined flavor defect, occurs sometimes and is then a problem to canners. Preliminary observations have indicated that the strong flavor of rutabaga is due mostly to its odor, contributed by the volatile components. An investigation was undertaken to isolate, characterize, and identify as many volatile components as possible in order to gain a better understanding of the chemical nature of rutabaga flavor.

MATERIALS AND METHODS

Materials. Rutabaga used were of the Laurentian variety, grown in medium-loam soil in Cumberland, Wisconsin, and harvested in October, 1962. A portion was stored at 10°C, and another portion was canned. Preliminary work had been done in 1961 on rutabaga from the same source, harvested in the fall.

Methods. *Canning procedure.* Rutabaga was peeled with an abrasive peeler, trimmed, cut, and diced to ⅜-inch cubes. It was steam blanched at approximately 99°C for 7 min in a continuous blancher, hand packed in 303 × 407-size C-enamel-lined cans, covered with hot brine containing ½% salt and 1% sugar, processed 25 min at 121°C, and water cooled in the retort. The cans were stored at 6°C.

Precipitation methods. Carbonyl and sulfur compounds from freshly cooked rutabaga were respectively isolated as 2,4-dinitrophenylhydrazones (DNPH) and mercury salts. A slurry of rutabaga was prepared by dispersing a weighed amount of freshly ground rutabaga with approximately an equal amount of distilled water in a blender. It was placed in a 4-L 2-neck Pyrex round-bottom flask jacketed with a heating mantle. One neck of the flask was fitted with an adapter that mounted an inlet glass tubing extended to about 1 inch from the bottom of the flask, whereby nitrogen could be bubbled through the slurry. The other neck was fitted with a 20-cm reflux con-

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denser. Leading from the condenser were series of drying U-tubes and/or 500-ml gas-washing bottles containing one or more of the following chemicals: a) anhydrous calcium chloride, b) lead acetate, c) 4% mercuric cyanide solution, d) 3% mercuric chloride solution, and e) 0.2% 2,4-dinitrophenylhydrazine (DNP) in 2*N* HCl to absorb water, hydrogen sulfide, mercaptans, mono- or disulfides, and carbonyl compounds, respectively. The slurry was heated to boiling in about 30 min and held simmering for 20 hr. Nitrogen gas was flushed at a rate of 45–60 ml per min. At the end of the process, the traps were held at 2°C for 48 hr. Precipitates were collected and dried in a vacuum desiccator over calcium chloride.

Carbonyl compounds. A portion of the precipitate (DNPH) (hydrozone) collected from the 2,4-dinitrophenylhydrazine absorption trap described above was stirred with a generous amount of boiling ethyl alcohol to separate the hydrazones into mono- and polycarbonyl fractions (Pippen *et al.*, 1958). Column chromatography by systems described by Gordon *et al.* (1951) and Spencer and Stanley (1954) was employed to separate the monocarbonyl fraction further. Melting points and mixed melting points of authentic and of isolated DNPH were determined with Fisher-Jones melting-point apparatus. Visible and infrared absorption spectra were respectively obtained with a Beckmann DK-2 spectrometer and a Beckman IR-4 infrared spectrometer. Paper chromatographic techniques described by Huelin (1952), using Whatman No. 1 paper and a methanol-heptane (1:2 v/v) solvent system, and by Lynn *et al.* (1956), using Whatman No. 1 paper impregnated with 2-phenoxyethanol (15% in acetone) and developed with heptane, were used for examination of the carbonyl compounds.

Sulfur compounds. 1) *Mercury salts of sulfur compounds.* Portions of the precipitate collected from the mercuric cyanide absorption trap were treated with 4*N* HCl to detect the presence of hydrogen sulfide and/or mercaptans. Portions of the precipitates from the mercuric chloride absorption traps were treated with either 4*N* HCl or 10% NaOH to detect the presence of disulfide and monosulfide, respectively. Sulfur compounds were regenerated by heating gently another portion of this precipitate with 5*N* HCl and collecting in mercuric salt absorption traps or in a series of condensing traps cooled by ice water and liquid nitrogen. The content of the latter was examined by gas chromatographic techniques.

2) *Quantitative determination of hydrogen sulfide.* Hydrogen sulfide evolved from cooking fresh or canned rutabaga was quantitatively estimated by the methylene blue method described by Sands *et al.* (1949). The brine or slurry of rutabaga was heated in a 500-ml 2-neck round-bottom flask equipped with a 20-cm reflux condenser and brought to boiling in 5–7 min. The noncondensables were flushed by nitrogen into a 2% zinc acetate solution, replaced hourly or whenever turbidity developed. The samples, procured over a period of 8 hr, were properly diluted and the color developed. The optical density of the samples at 665 mμ was measured in duplicate with a Bausch and Lomb Spectronic 20 colorimeter. Readings were converted into hydrogen sulfide values by interpolation on a standard curve prepared with sodium sulfide as described by Marbach and Doty (1956).

Basic components of rutabaga. A slurry of fresh rutabaga was adjusted to pH 10.5 with 1*N* NaOH and subjected to steam distillation for 3 hr at the rate of about 700 ml of distillate per hr. The distillate was titrated with HCl to a phenolphthalein end point and evaporated to dryness. The residue was washed several times with ethyl ether and dried.

Small portions of the residue were treated with a few drops of 10% NaOH. The odor of the volatiles released, the reaction with litmus paper, and behavior on exposure to concentrated HCl were observed. A Nessler reagent test for NH_3, the ninhydrin test, and the carbylamine test (Linstead and Weedon, 1956) were performed to detect primary amine.

Detection of isothiocyanates in fresh rutabaga. Isothiocyanates were isolated as thioureas from fresh rutabaga by the method described by Stahmann *et al.* (1943). The thioureas were extracted with 95% ethyl alcohol. A portion was further diluted with 95% ethyl alcohol for spectrometric examination and for the Grote reagent test (Grote, 1931). Another portion was concentrated by partial removal of ethyl alcohol for paper chromatographic examination using a chloroform-water system described by Kjaer and Rubinstein (1953).

Preliminary study of volatile components of rutabaga by gas chromatography. 1) Isolation of *volatile components.* a) *Solvent extraction method.* Approximately 500 ml of distillate were collected from the steam distillation of rutabaga slurry, prepared in a blender with the contents of two cans (about 850 g, including brine) of rutabaga. The distillate was extracted repeatedly in a separatory funnel with 200-, 100- and 50-ml portions, successively, of anhydrous ethyl ether. The combined ether extract was dried by the addition of anhydrous sodium sulfate and concentrated to 10–15 ml, and portions were examined by gas chromatography. A slurry was prepared with 500 g of fresh instead of canned rutabaga, plus 500 ml of distilled water. Ether extraction of the steam distillate followed.

b) *Direct trapping method.* A slurry of rutabaga was prepared in a blender with the contents of two cans (about 850 g, including brine) of rutabaga plus 500 ml of distilled water, and placed in a 2-L two-neck round-bottom flask. One neck was fitted with an adapter for the introduction of high-purity nitrogen, and the other was fitted with a 20-cm reflux condenser. Leading from the condenser was a series of three traps, respectively submerged in ice water, dry ice-acetone, and liquid nitrogen. The first two were 24 × 140 mm; the last trap was 10 × 100 mm, packed with glass wool to provide more surface for condensing volatiles, similar to that designed by Thomson (1961). The slurry was heated to boiling in about 20 min and kept simmering. High-purity nitrogen was flushed at a rate of 35–40 ml per min. The liquid-nitrogen trap was replaced hourly; the dry ice-acetone trap was retained for 4 hr. The volatiles collected were warmed to room temperature and later examined by gas chromatography.

2) *Examination of volatile components by gas chromatography.* A Barber Coleman Model 10 gas-liquid chromatograph with a tritium ionization detector was used to examine the volatile components isolated from rutabaga. Pyrex U-shaped glass columns, each 2.44 m long and of 5 mm inside diameter were used with various organic packings. Liquid samples were introduced by Hamilton microsyringe to the column directly. Gaseous samples were introduced through a loop system similar to that described by van de Craats (1956). The time of entry through the loop system was determined and used for correcting retention times of unknown samples.

RESULTS AND DISCUSSION

Precipitation methods. A black reaction product was obtained in the lead acetate trap, indicating the presence of hydrogen sulfide. No precipitate was observed in the mercuric cyanide absorption trap, indicating the absence of mercaptan; white precipitate appeared in the mercuric chloride absorption trap, indicating the presence of monosulfide(s) and/or disulfide(s); yellow precipitate appeared in the DNP absorption trap, indicating the presence of carbonyl compounds.

In the semiquantitative experiments the calcium chloride and lead acetate traps were eliminated; the mercuric cyanide absorption trap was used to absorb hydrogen sulfide because of the apparent absence of mercaptan. In all cases, a train of five traps was used. The arrangement of the traps and results are summarized in Table 1.

Variations were obtained in the amounts of precipitates from runs I and II of freshly cooked rutabaga. The differences were possibly due to inherent differences in the rutabaga samples, since the experimental conditions were controlled as far as possible. The position of the traps was found to be important. The precipitate in DNP traps in run II increased fourfold over that in run I, when their position was changed from fourth and fifth to first and second; thus, canned rutabaga was used in run III. The amount of precipitate formed in the mercuric cyanide trap was greater, the amounts of the precipitates in the mercuric chloride and DNP traps less than in the corresponding traps in run II used for freshly cooked

Table 1. Isolation of volatiles of rutabaga as precipitates (3 runs).

	I Fresh			II Fresh			III Canned		
Weight	1,200 g			1,000 g			1,100 g		
Moisture	88.6%			89.5%			91.1%		
Position of traps	(1) 4% $Hg(CN)_2$			(1) 0.2% DNP			(1) 0.2% DNP		
	(2) 3% $HgCl_2$			(2) 0.2% DNP			(2) 0.2% DNP		
	(3) 3% $HgCl_2$			(3) 4% $Hg(CN)_2$			(3) 4% $Hg(CN)_2$		
	(4) 0.2% DNP			(4) 3% $HgCl_2$			(4) 3% $HgCl_2$		
	(5) 0.2% DNP			(5) 3% $HgCl_2$			(5) 3% $HgCl_2$		
Rate of N_2 flushing	45–60 ml/min			45–60 ml/min			45–60 ml/min		
Time of N_2 flushing	20 hr			20 hr			20 hr		
Wt. of ppt. collected	mg	mg/kg		mg	mg/kg		mg	mg/kg	
	total	wet	dry	total	wet	dry	total	wet	dry
$Hg(CN)_2$ trap	47	39	4.5	80	80	8.4	198	180	16.1
$HgCl_2$ trap	297	247	28.2	320	320	33.6	245	232	20.8
DNP trap	52	44	5.0	115	115	20.5	145	132	11.8

rutabaga. After the slurry was simmered for 20 hr, further formation of precipitates appeared negligible. While not strictly quantitative, the results indicated the amounts of precipitates that could be obtained from cooked rutabaga.

Carbonyl compounds. Acetaldehyde was identified to be the major constituent in carbonyl compounds, isolated as 2,4-DNPH from freshly cooked rutabaga. Polycarbonyl compounds were not detected in the isolated DNPH, since the latter is quite soluble in hot ethyl alcohol and did not give a blue-violet color in ethanolic KOH (Pippen *et al.*, 1958).

The isolated DNPH had a melting range of 147–158°C. Attempts were made to fractionate the isolated DNPH further by column chromatography, using the two systems previously described. In both, only one band and a very slight stationary zone were observed upon development. A mixture of known hydrazones was readily separated by each of the two systems. This was taken as evidence there could be only one major compound in the isolated DNPH. Paper chromatography with two systems further confirmed that there was only one spot after development with an R_f value the same as that of authentic acetaldehyde 2,4-DNPH. The infrared spectrum of the isolated DNPH was identical with that of acetaldehyde 2,4-DNPH. The visible absorption spectra of both the isolate and authentic acetaldehyde derivative showed maximum absorption at 355 mμ in ethyl alcohol and a major peak at about 425 mμ in ethanolic KOH. Acetaldehyde isolated from bread as DNPH was reported to have a melting range of 158.5–168°C (Ng *et al.*, 1960). The range may have been due to traces of impurities, or because acetaldehyde DNPH exists in several crystalline modifications; melting-point values reported have been from 147 to 170°C (Braddock *et al.*, 1953).

Sulfur compounds. *Mercury salts of sulfur compounds.* A yellowish-green precipitate was obtained in the mercuric cyanide absorption trap from volatile components of freshly cooked rutabaga, indicating the presence of both hydrogen sulfide and mercaptan(s). Hydrogen sulfide reacts with aqueous mercuric cyanide to form a black HgS precipitate; mercaptans react with aqueous mercuric cyanide to form white $Hg(SR)_2$ or $Hg(SR)_2 \cdot Hg(CN)_2$ precipitates. When both hydrogen sulfide and mercaptan are present, the color of the precipitate with aqueous mercuric cyanide could be yellow, yellowish-green, or black, depending on the relative quantities of the two compounds (Challenger, 1959). If warmed with dilute acid, mercaptan will be liberated, leaving HgS essentially unaltered. A portion of the precipitate from the mercuric-cyanide-absorption trap was warmed with 4*N* HCl, and a faint sulfurous odor was detected. However, when another portion of the precipitate was warmed with 4*N* HCl and flushed continuously for 10 hr with high-purity nitrogen into mercuric cyanide, no precipitate was observed. This indicated that mercaptan was not present in an amount sufficient to be detected by precipitation, although it could be detected by smell upon regeneration. Apparently the precipitate in the mercuric cyanide absorption trap was principally HgS from hydrogen sulfide evolved during the cooking of fresh rutabaga.

A white precipitate was obtained in the mercuric chloride absorption traps from volatile components of freshly cooked rutabaga. This indicated the presence of monosulfide (RSR) and /or disulfide (RSSR). When a portion of the precipitate was treated with 10% NaOH and warmed, a sulfurous odor was detected, indicating the presence of monosulfide(s). When another portion was warmed with 4*N* HCl, a strong odor similar to that of mercaptan was also perceived, indicating the presence of disulfide(s). It appears that the precipitate could have been a mixture of monosulfide and disulfide. A portion of the precipitate was heated gently with 5*N* HCl under reflux. The regenerated sulfur compounds were flushed with N into a series of two traps, one containing 4% mercuric cyanide solution to absorb mercaptan, and the other 3% mercuric chloride solution to absorb monosulfide. Small quantities of precipitates were obtained in both traps. The quantities of precipitates were very small, and further identification of the compounds

was not possible. By gas chromatography, two peaks were shown when the regenerated sulfur compounds in the liquid-nitrogen trap were examined in columns with dinonyl phthalate and $\beta'\beta'$-oxydipropionitrile, respectively. The retention times of the unknown peaks and of known compounds are summarized in Table 2. Peaks 1 and 2 were respectively identified as methyl mercaptan (CH_3SH) and dimethyl sulfide (CH_3SCH_3). The odors of the unknown and of known samples emerging from the column were similar. Methyl mercaptan presumably developed from fission of methyl disulfide in mercuric chloride solution (Challenger, 1959). It was concluded that dimethyl sulfide (CH_3SCH_3) and methyl disulfide (CH_3SSCH_3) were present in the volatile components of freshly cooked rutabaga.

Volatile sulfur compounds have been found in many vegetables, particularly in the genus *Brassica,* such as cabbage, and in the genus *Allium,* such as onions and garlic. It is generally accepted that sulfur compounds contribute significantly to the characteristic flavor of vegetables of these genera. The precursor of methyl disulfide in cabbage had been shown to be L-*S*-methyl cysteine sulfoxide (Dateo *et al.,* 1957). This compound was also detected by Synge and Wood (1956) in vegetables of the genus *Brassica,* such as turnip, kale, white mustard, and others. This compound has not been previously reported in rutabaga. Presumably it could be a precursor of the dimethyl disulfide. A methyl methionine sulfonium salt was isolated by McRorie *et al.* (1954) from cabbage juice and detected in parsley, lettuce, turnip greens, and other vegetables. It was considered to be a possible precursor of dimethyl sulfide. Dimethyl sulfide was identified in rutabaga in this study. It was not detected in cooked cabbage by Dateo *et al.* (1957). Methyl methionine sulfonium salt has not been reported in rutabaga. It could be a possible precursor of dimethyl sulfide.

Quantitative determination of hydrogen sulfide. Hydrogen sulfide was estimated by the methylene blue method to be 2.07 mg/kg of fresh rutabaga, 0.46 mg/kg drained weight of canned rutabaga, and 0.77 mg/L of brine from canned rutabaga in an 8-hr boiling. Hydrogen sulfide forms during the cooking of fresh rutabaga. When fresh rutabaga was heated to and maintained at about 70°C and flushed with nitrogen for 9 hr, only a trace of H_2S was subsequently detected by the methylene blue method.

Basic components of rutabaga. The volatile regenerated from the salt isolated from the basic steam distillation of fresh rutabaga was identified as ammonia by the following: a) a few drops of a 10% NaOH were added to a portion of the unknown salt; an odor of ammonia could be perceived; the vapor evolved turned red litmus paper to blue; b) a white "cloud," supposedly NH_4Cl, was formed when a stirring rod moistened with concentrated HCl was exposed to the mouth of the flask containing a portion of the unknown salt to which 10% NaOH was added; c) a portion of the unknown salt plus a few drops of 10% NaOH gave an orange precipitate when Nessler's reagent was added. Tests *a* and *b* did not exclude the possibility of the presence of primary amines such as methylamine. The response was negative with the carbylamine (no offensive odor) and with the ninhydrin tests. It was concluded that primary amine was not present. A yield of approximately 1 meq of basic components

Table 2. Comparison of retention times (min) of regenerated sulfur compounds from mercury salts obtained from mercuric chloride absorption traps, and of known compounds.

Unknown Peak	Dinonyl phthalate			β,β'-oxydipropionitrite			Known compounds
	Unknown			Unknown			
	Uncorrected	Corrected[a]	Known[b]	Uncorrected	Corrected	Known	
1	2.2	2.0	2.0	5.8	5.6	5.6	CH_3SH
2	3.2	3.0	3.0	7.8	7.6	7.6	CH_3SCH_3

[a] Corrected for the time required for an unknown sample to pass through a loop system prior to entering column.
[b] Sample introduced directly into column.

or about 17 mg ammonia per kg fresh rutabaga was obtained.

Detection of isothiocyanates in fresh rutabaga. The presence of isothiocyanates in the steam distillate of fresh rutabaga was detected by the conversion of isothiocyanates to thioureas. The ethyl alcohol extract of the residue obtained by a low-temperature evaporation of the ammonia-treated distillate of fresh rutabaga gave a deep blue color with Grote reagent in about 2 min, indicating the presence of thiourea(s) (Grote, 1931). The extract had maximum absorption at 243 mμ (Fig. 1), which is

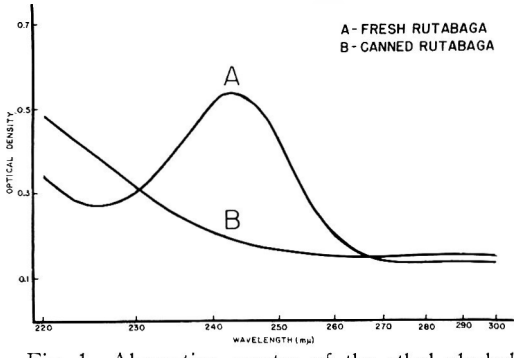

Fig. 1. Absorption spectra of the ethyl alcohol extracts of residues obtained from low-temperature evaporation of steam distillates of fresh and canned rutabaga, each collected in ammonia hydroxide solution.

characteristic for thiourea (Kjaer *et al.*, 1953). Paper chromatography, using the chloroform-water system, was applied to separate the isolated crude thiourea(s). Two spots, one with an R_f value of 0.8 and the other at the origin, were observed after developing and spraying with Grote reagent. No further attempts were made to identify the components. Isothiocyanates have been detected in many plants and were considered to be of importance in contributing to the pungent odor of vegetables in the Cruciferae family. A similar experiment was conducted on about 1 kg (dry wt.) of canned rutabaga. The alcohol extract gave a faint blue color with Grote reagent. It did not have maximum absorption in the ultraviolet region of 220–340 mμ (Fig. 1). This could have been due to presence of impurities that altered the characteristic absorption pattern or the absence of thiourea derivatives. Isothiocyanates are believed to be released from corresponding thioglucosides by enzymic reaction during the maceration of plant tissue. Enzymes, however, were supposedly inactivated during heat treatment of the rutabaga. It could be speculated that isothiocyanates could not be present in any significant quantity in canned rutabaga.

Preliminary study of volatile components of rutabaga by gas chromatography. Gas chromatographic techniques were used to supplement the chemical methods of analysis and to detect other components. Two isolation procedures were used: solvent extraction and direct trapping of volatiles. Typical chromatograms obtained with the solvent extraction procedure are shown in Fig. 2. Ten peaks were shown in the chromatogram of the other extract of steam distillate of canned rutabaga (Fig. 2A), and eleven peaks were shown in that of the fresh rutabaga (Fig. 2B), when examined in a column with polyethylene glycol 1540. The retention times of the components are summarized in Table 3.

The retention times of the compounds from fresh and canned rutabaga appeared to be comparable, except for peak 3, not shown in the chromatogram of canned rutabaga (Fig. 2A). This peak could have been obscured by peak 2. Peaks 1 and 2 in Figs. 2A and 2B were very close to the ether peak, which would make subsequent isolation of individual peaks for further identification difficult. Chloroform, hexane, dioxane, and isopentane were used as extraction solvents in place of ethyl ether; only isopentane was found to be suitable. Isopentane extract of the steam distillate of canned rutabaga showed 12 peaks when examined in the column with dinonyl phthalate (Fig. 2C). The volatiles collected in the liquid nitrogen traps were examined in each of eight columns, of which β,β′-oxydipropionitrite column was the most satisfactory. Squalane column gave moderately good separation for components with high retention times.

The volatiles collected during simmering of the rutabaga slurry for two hours gave the same qualitative results as simmering for 3–4 hr. A typical chromatogram of volatiles from canned rutabaga collected in the liquid nitrogen trap is shown in Fig. 3. Twelve peaks are shown; the respective re-

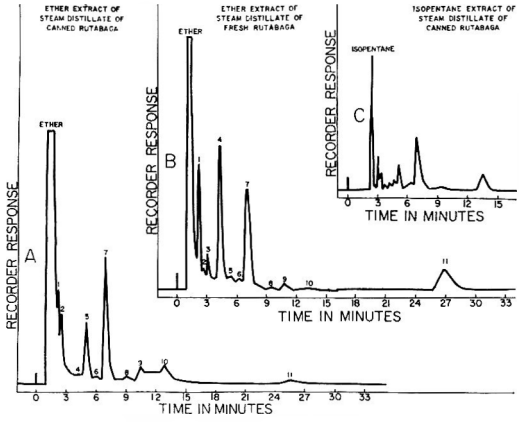

Fig. 2. Gas chromatograms.

A. Ether extract of steam distillate of canned rutabaga
Conditions:
Column: 24 m × 5 mm, 20% polyethylene glycol 1540 on 80–100-mesh "Embacel" kieselguhr
Carrier gas: argon
Flow rate: 55 ml/min
Pressures: inlet, 10 psig; outlet, atmospheric
Temperatures: column, 98°C; detector, 129°C; flash heater, 138°C
High voltage: 1,000 volts
Sensitivity: 30
Sample size: 5 μl

B. Ether extract of steam distillate of fresh rutabaga
Conditions:
Same as in A

C. Isopentane extract of steam distillate of canned rutabaga
Conditions:
Column: 24 m × 5 mm, 20% dinonyl phthalate on 60–80-mesh "Embacel" kieselguhr
Carrier gas: argon
Flow rate: 70 ml/min
Pressures: inlet, 10 psig; outlet, atmospheric
Temperatures: column, 98°C; detector, 127°C; flash heater, 138°C
High voltage: 1,000 volts
Sensitivity: 10
Sample size: 3 μl

tention times of peaks 1 to 12 were 1.9, 3.0, 3.6, 5.8, 7.4, 9.0, 10.7, 13.1, 15.5, 19.1, and 37.9. From the retention times of known compounds under the same conditions and from the odors emerging from the chromatographic column, a number of compounds were tentatively identified (Table 4).

These compounds—except methyl mercaptan, only a trace of which was detected—were identified in the volatiles of freshly cooked rutabaga by chemical precipitation methods. The retention time of known methyl disulfide was 1.7 min longer than that of peak 12, which was over 30 min. Under the experimental conditions used, retention times over 30 min were not always very precise when using the column with β,β′-oxydipropionitrite. Peak 12 had a sulfurous odor when emerging from the column. Since methyl disulfide was previously identified by chemical methods, peak 12 was tentatively assigned as methyl disulfide.

A comparison of the chromatograms of volatiles from canned rutabaga collected in the liquid nitrogen traps hourly during 4 hr showed that peak 1 was absent, peaks 4 and 8 were reduced, and peaks 3 and 10 were increased, after the first hour of simmering. The chromatogram of the volatiles collected in the dry ice-acetone trap during 4 hr showed fewer peaks, every one of which was present in the volatiles collected in the liquid nitrogen trap.

The volatile sulfur compounds were found to contribute significantly to the strong, pungent odor characteristic of rutabaga. Steam distillate of fresh rutabaga slurry had a strong and characteristic odor. When the distillate was collected in a solution of mercuric chloride, which absorbed the volatile sulfur compounds, the original strong

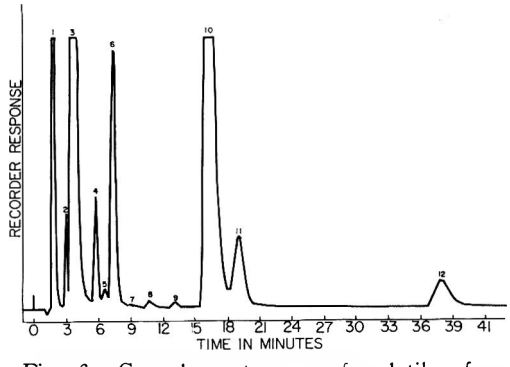

Fig. 3. Gas chromatogram of volatiles from canned rutabaga collected in trap submerged in liquid nitrogen.
Conditions:
Column: 24 m × 5 mm, 23% β,β′-oxydipropionitrite on 80–100-mesh Gas-Chrom P
Carrier gas: argon
Flow rate: 65 ml/min
Pressures: inlet, 10 psig; outlet, atmospheric
Temperatures: column, 54°C; detector, 125°C: flash heater, 118°C
High voltage: 1 000 volts
Sensitivity: 30
Sample size: about 15 ml

rutabaga odor was reduced markedly. When the steam distillate was collected in DNP solution, which removed the volatile carbonyl compounds, the rutabaga odor was modified to some extent; the strong, pungent odor was not reduced appreciably. This suggests that the carbonyl compounds were not as important as the sulfur compounds in contributing to rutabaga flavor. Pattan and Josephson (1957) reported that methyl sulfide, methyl disulfide, methyl mercaptan, and acetaldehyde have respective threshold values of 0.012, 0.021, 0.002, and 1.3 ppm. It is likely that the presence of "bitterness" in rutabaga could be due to high concentrations of sulfur compounds.

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Volatile Esters of Bartlett Pear. III.[a]

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SUMMARY

Bartlett pear essence was separated by gas chromatography without losing its desirable aroma characteristics. This was done by room-temperature on-column injections, followed by a programmed temperature increase of the chromatographic column. Evidence is presented indicating that the methyl (or other short-chain alcohol moiety) ester of *trans*-2:*cis*-4 decadienoic acid is present. Hexyl acetate was identified as a "contributory flavor compound" of Bartlett pear. The infrared spectra of several other esters, which may be "character impact compounds," are presented, and their correlations with functional groups are discussed.

A ten-carbon diunsaturated straight-chain fatty acid has been reported (Jennings, 1961; Jennings and Creveling, 1963) among the hydrolysis products of Bartlett pear esters. Other components of this mixture included ethyl, *n*-propyl, *n*-butyl, *n*-amyl, and *n*-hexyl alcohols, and acetic, *n*-butyric, *n*-caproic, *n*-caprylic, *n*-pelargonic, and *n*-capric acids.

This paper reports on chromatography of the pear assence esters as such, and subsequent characterization of collected fractions.

METHODS

Gas chromatography. Used for analytical chromatography was an Aerograph, Model 600 B, Hy-Fi, with ⅛-inch × 10-ft stainless-steel columns packed with 10% Carbowax 20M on 60–80 Chromosorb P. These runs were isothermal. Preparative-scale gas chromatography utilized an Aerograph, Model A-700, chromatograph, with a ⅜-inch × 20-ft aluminum column packed with 15% Carbowax 20M on 45–60 Chromosorb P. Runs were normally programmed from ambient temperature to 250°C. Collections were in Pyrex traps immersed in ice water. Isolated fractions were reinjected on a ¼-inch column packed with 10% silicone grease on Chromosorb P and recollected in chilled Pyrex tubes for infrared analysis.

Infrared spectroscopy. Infrared spectra were determined with a Beckman IR-5 infrared spectrophotometer. Undiluted samples were sandwiched between two NaCl plates, poised as far from the source compartment as possible.

RESULTS AND DISCUSSION

A major problem in preparative-scale gas chromatography of pear essence has been apparent thermal degradation in the injection chamber. Previous atempts had utilized a commercial instrument designed for large sample injections. After rapid vaporization in a high-heat density injection chamber, the volatiles were conducted through a manifold to a system of columns in parallel. Recombined chromatographed fractions no longer possessed the typical Bartlett pear aroma. The present study used a commercial instrument designed for multiple small injections. With a long needle, samples were inserted through the injection port and deposited directly on the column packing at room temperature. The column was then heated rapidly and the material was volatilized on-column and in the helium stream. The recombined chromatographed fractions still possessed the desired aroma of Bartlett pear. Comparison of the recombined chromatographed fractions with the unchromatographed essence on an analytical ⅛-inch column with flame ionization detection showed some differences in the relative amounts of various fractions, but these can probably be attributed to variations in efficiency in trapping the different fractions. No new peaks were found, and all peaks shown by the original essence also occurred

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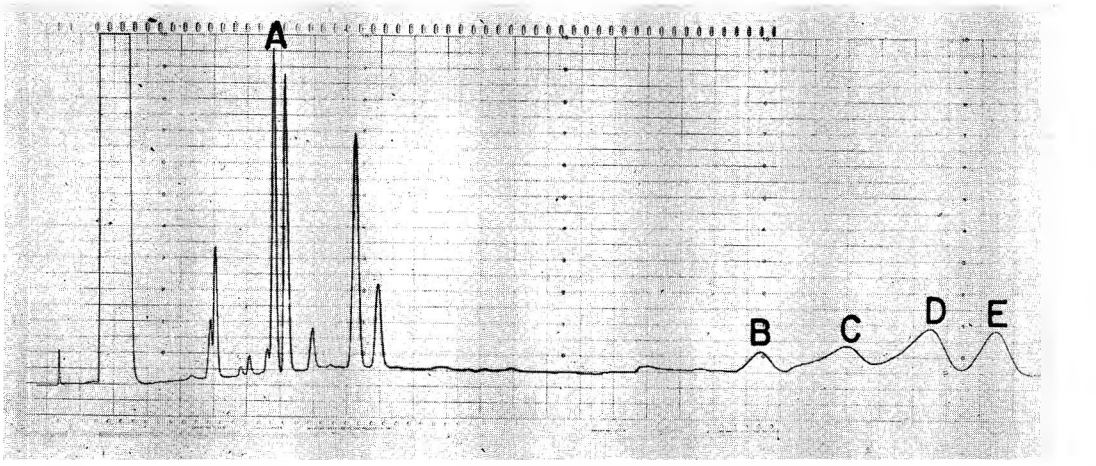

Fig. 1. Preparative-scale chromatogram of Bartlett pear essence. Injection, 0.5 ml on column at room temperature. Heaters turned on, and temperature increased continuously to 250°C at end of chromatogram.

in the recovered essence. Consequently, it would appear that on-column injections performed in this manner permit isolation of individual essence fractions with some assurance that subsequent sensory evaluation or chemical characterization studies are actually being performed on unaltered pear constituents. To ensure that these samples were not contaminated by bleed-off from the Carbowax collection column, they were re-chromatographed on a ¼-inch column packed with 10% silicone grease on Chromosorb P just prior to infrared spectral analysis. The chromatograms from these collections indicated a degree of overlap in some of the preparative-scale collections. As a final check on purity, spectro-grade carbon tetrachloride was used to recover the samples after infrared analysis, and the homogeneity of the recovered material (disregarding the CCl_4 peak) was verified by analytical gas chromatography.

Meaningful flavor chemistry must be correlated with sensory evaluation studies, but, to date, use of the latter technique in this work has necessarily been restricted. Valid sensory evaluation procedures involve replicate testing by several judges, and the total sample requirement is about ½ ml. Chemical characterizations, in contrast, can utilize samples of only about 1 μl. Because of the limited amounts of material available, it seemed more logical to utilize the restricted amounts of isolated fractions available to establish structure, with the goal of synthesizing these materials to make larger quantities of the synthetic substances available to the sensory evaluation panels. In connection with the chemical studies, however, attention is given to the aromas of individual traps, and after the sample has been removed from the trap (with a microsyringe or glass capillary) the emptied trap is occasionally delivered to the sensory evaluation laboratories for their opinions and discussion. Although this cannot be regarded as a statistically valid procedure, and many of the determinations and descriptions are made by individuals whose acuity for the odor in question has not been tested, some conclusions can be drawn.

It would appear that, with regard to sensory evaluation, aroma compounds from these natural sources can frequently be divided into two separate classes. One class, consisting of natural components of the substance in question that individually possess aroma suggestive of and differentiating that substance, might be termed "character impact compounds." The other class might be termed "contributory flavor compounds." These could be defined as natural components of the substance in question that contribute fruitiness, bouquet, or fullness but do not, by themselves, suggest that particular fruit or source product. For example, acetaldehyde is almost surely a contributory flavor compound for strawberries, oranges,

pear, and most other fruits. Alone, it doesn't possess the aroma of these fruits, but if it were eliminated from the natural fruit essence its absence would be noticeable.

Fig. 2 (top) shows the infrared spectrum of a contributory flavor compound isolated from pear essence. Most individuals have described the aroma as banana-like, or fruity. Only one individual recognized it as coming from pear. The strong absorption at 1740 cm^{-1} is best attributed to the C = O stretch of a saturated, aliphatic ester (Bellamy, 1958; Silverstein and Bassler, 1963). The absorption at 1240 cm^{-1}, generally attributed to ester C—O— stretch as modified by the acid moiety, suggests that this is an acetate ester. Comparison of its retention on the gas chromatograph with retentions of a series of known acetate esters established that this fraction had the same retention as hexyl acetate on DEGS and on silicone grease. Accordingly, hexyl acetate was synthesized by refluxing hexyl alcohol and glacial acetic acid. The crude ester was separated by distillation, and purified by gas chromatography. Its spectrum (bottom, Fig. 2) agrees precisely with that of fraction A.

Recovery of some of the other fractions indicated in this chromatogram was complicated by their strong tendency to fog, resulting in low trapping efficiencies. Although polyester columns such as the Carbowax 20M do an excellent job of resolving this essence mixture, the accompanying column bleed necessitates a second pass through a non-bleeding column prior to infrared analysis. Aerosol loss in the two chromatographic passes reduced many of these fractions to amounts too small for further analysis. Attention is now being directed to designing a Volman trap (Teranishi *et al.*, 1962) adaptable to these chromatographs, and study of these fractions will be resumed when fresh lots of pear essence are available.

Fractions B, C, D, and E were the major high-boiling fractions collected. During the period that these fractions were emerging from the chromatograph, the laboratory was blanketed with a pungent, pear-like aroma reminiscent of cooked pear, spiced pear, or pear jam. Each of the recovered high boilers possessed a similar aroma, suggesting that all were character impact compounds, or that each contained a common

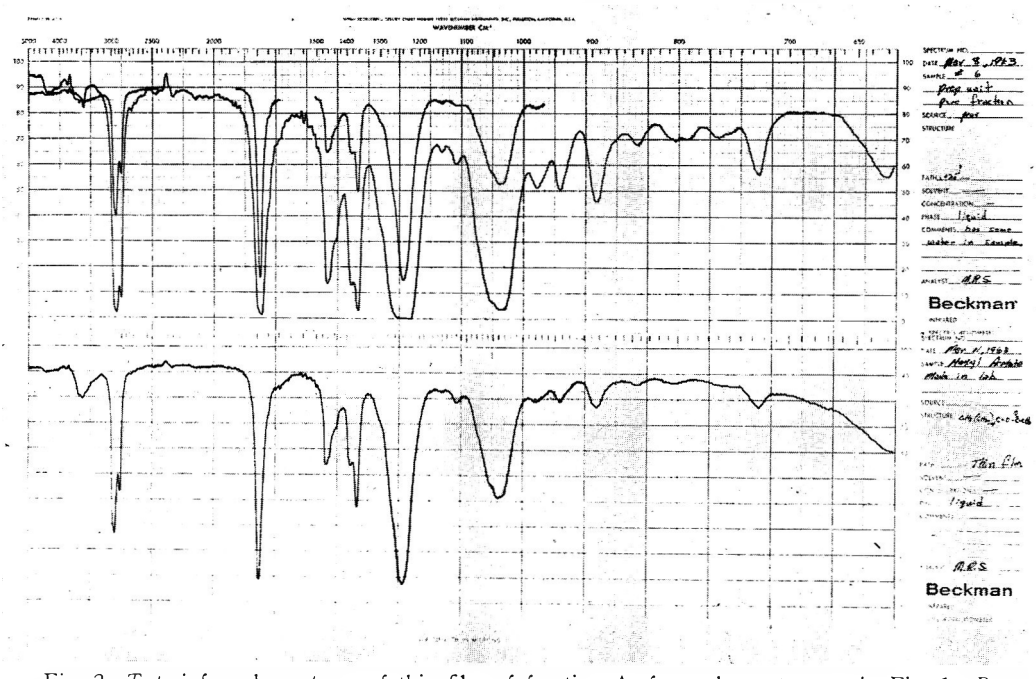

Fig. 2. *Top,* infrared spectrum of thin film of fraction A, from chromatogram in Fig. 1. *Bottom,* infrared spectrum of thin film of synethic hexyl acetate.

substance emanating from the column for a considerable period, which was responsible for the common aroma. In either case, it is quite possible that the flavor resulting from heat, normally described as "cooked," is not necessarily due to heat-formed compounds. Heat may reduce the concentration of the more volatile low boilers, so that more of the remaining flavor is due to the high boilers and their characteristic "cooked" flavor.

A considerable amount of useful information can be derived from the infrared spectrum of compound B (Fig. 3). This is certainly an ester (absorptions at 1720, 1266, and 1165 cm^{-1}), but the shift of the carbonyl absorption to 1720 cm^{-1} indicates a structure resembling $-C=C-\overset{O}{\overset{\|}{C}}-O-$ (Bellamy, 1958; Nakanishi, 1962). Additional conjugated unsaturation could be present without causing a noticeable further shift of this absorption. The absorptions at 1639 and 1610 cm^{-1} also indicate unsaturation, and that at 995 cm^{-1} is suggestive of a *trans* configuration (Bellamy, 1958). The absorption at 965 cm^{-1} is somewhat weaker than would normally be expected in light of this, but it might be ascribed to resonance patterns in the molecule. There are indications that fraction B is a methyl ester (1165 cm^{-1}; Nakanishi, 1962). The strength of the absorption at 1639 cm^{-1} indicates asymmetric location of the unsaturation (O'Connor, 1956). All of these correlations could be satisfied by esters of 2,4-decadienoic acid, which Jennings and Creveling (1963) previously isolated from the hydrolysate of Bartlett pear essence. Crombie (1955) studied the stereochemistry of several conjugated dienes, including the four isomeric 2,4-decadienoic acids and their methyl esters. Table 1 shows his major infrared assign-

Table 1. Infrared assignments (cm^{-1}) for stereoisomeric methyl 2,4-decadienoates.[a]

Isomer	$C_2=C_3$	$C_4=C_5$	difference
trans-2 : *trans*-4	1647	1618	29
trans-2 : *cis*-4	1639	1607	32
cis-2 : *trans*-4	1640	1602	38
cis-2 : *cis*-4	1634	1592	42

[a]Crombie, 1955.

ments for the stereoisomeric methyl 2,4-decadienoates. The absorption spectra that he published are difficult to compare directly with transmission spectra currently available and those presented in this paper. It would appear that practically all absorptions—major, minor, and shoulders—exhibited by fraction B agree with his data for methyl *trans*-2:*cis*-4-decadienoate. We are currently synthesizing this material for further work.

Fig. 4 shows the infrared spectrum of fraction C. This, again, is an ester (absorptions at 1735, 1260, 1170 cm^{-1}). The absorption at 1640 cm^{-1} indicates unsaturation of the type C=C (O'Conner, 1956; Nakanishi, 1962), and because the carbonyl absorption remains at 1735 cm^{-1}, this is not

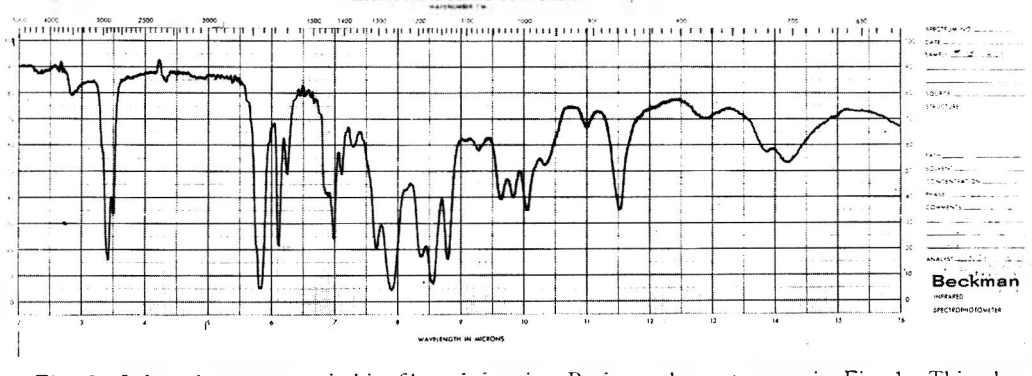

Fig. 3. Infrared spectrum of thin film of fraction B, from chromatogram in Fig. 1. This shows excellent agreement with the absorption spectrum of methyl *trans*-2:*cis*-4 decadienoate published by Crombie, 1955.

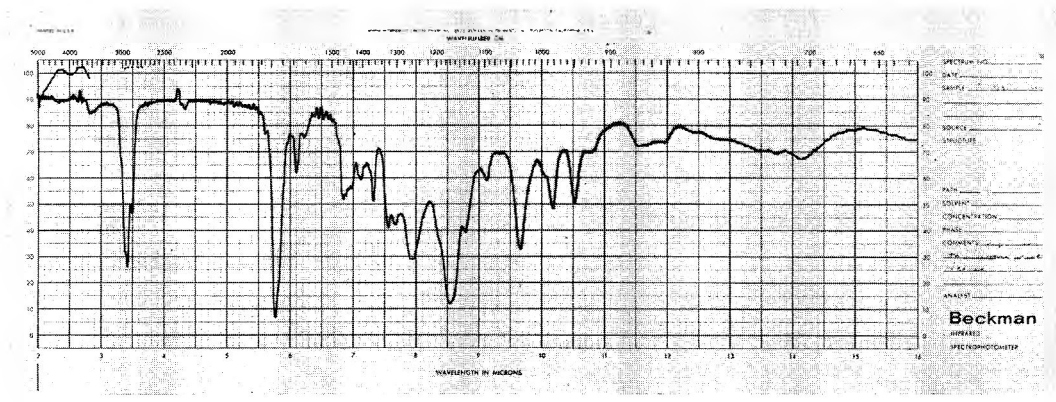

Fig. 4. Infrared spectrum of thin film of fraction C, from chromatogram in Fig. 1.

conjugated. These correlations imply that this is a monounsaturated ester. Because it exhibits a longer retention than that of the diunsaturated fraction B, the alcohol moiety of C is probably considerably larger than that of B (Lipsky *et al.*, 1959).

Fraction D, whose infrared spectrum is shown in Fig. 5, again appears to have many structural features in common with fraction B. The absorptions at 1720, 1265, and 1173 cm^{-1} indicate that this too is an ester, and the shift to 1720 correlates with conjugated unsaturation (Nakanishi, 1962).

The strong absorption at 869 cm^{-1} occurs in both fractions B and D, and in Crombie's spectra of the *trans*-2:*cis*-4 isomer only—and not in any of the other three isomers. We have been unable to find any published correlation for this absorption; it is probably due to the stereochemistry of the *trans*-2:*cis*-4 acid moiety, and suggests that fraction D is an ester of this same acid, with a higher-alcohol moiety accounting for its longer retention.

Subsequent chromatography of the material trapped as fraction E revealed that it was composed of approximately equal amounts of fractions D and E. Attempts to collect a pure sample of fraction E, whose trapping efficiency is apparently quite poor, resulted in recoveries too low for further analysis. Additional essence will have to be prepared before this study can be resumed.

It is probable that recognized pathways are involved in the biosynthesis of pear ester acids (e.g. Zill and Cheniae, 1962). Fruit maturation is accompanied by respiration, evidenced by evolution of CO_2 to the atmosphere and an increase of CO_2 in the flesh. Various investigators (Klein, 1957; Squires *et al.*, 1958; Gibson *et al.*, 1958) have studied the effects of HCO_3^-, which evidently stimulates the formation of fatty acids through increased incorporation of

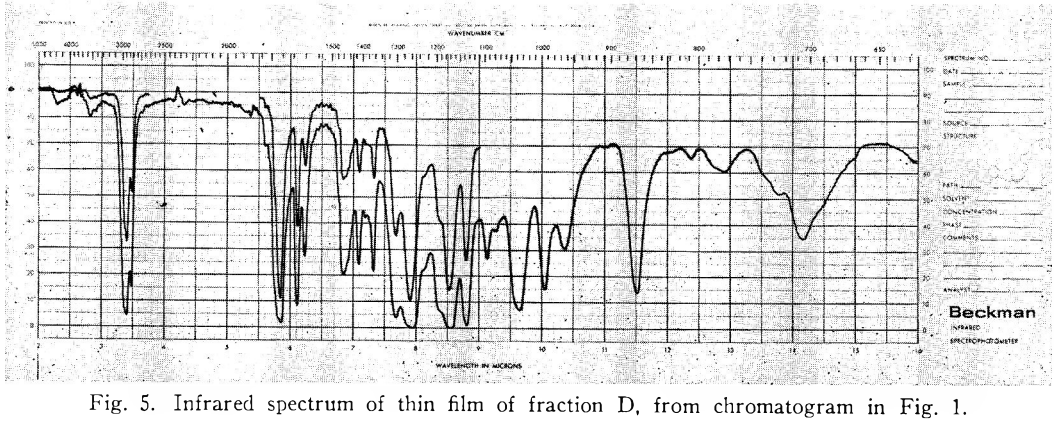

Fig. 5. Infrared spectrum of thin film of fraction D, from chromatogram in Fig. 1.

acetate (Wakil, 1958; Formica and Brady, 1959). Decadenoic acid could be visualized as resulting from carbohydrates or a coupling of lower-molecular-weight acids, but probably by a pathway resulting in direct formation of the diunsaturated acid, not by desaturation of the corresponding saturated acid (Hilditch, 1951; Simmons and Quackenbush, 1954; Painter, 1944). Subsequent esterification with the simple straight-chain alcohols could be postulated as involving enzymes or other co-factors, but the degree of ester formation and the type of ester formed would depend largely on the relative concentrations available of the various alcohols and acids.

The ideal maturation treatment for Bartlett pears is storage at −1 to 0°C for approximately 60 days, and subsequent ripening at 20°C. It has been established that this type of treatment results in fruit of maximum flavor (Claypool *et al.*, 1958; Leonard, 1963). Zill and Cheniae (1962) reported that low temperatures encourage the production of unsaturated fatty acids rather than their saturated counterparts, and that, under warmer conditions, the saturated fatty acids are produced. If the unsaturated esters isolated in this study do prove to be character impact compounds, it seems possible that the superior flavor developed by low-temperature storage could be attributed to increased production of unsaturated acids, and consequent higher concentration of their esters.

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The Determination and Contents of α- and γ-Tocopherols in Margarine

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SUMMARY

Methods are described for the determination of α-tocopherol and γ-tocopherol in margarine by column and paper chromatography and spectrophotometric measurement of the tocopherols. Thirty-seven samples of Norwegian margarine, average values 52 μg/g α-tocopherol and 89 μg/g γ-tocopherol, together with 18 samples from other European countries, were assayed. Ten of the samples were further assayed after 7 months of storage, and showed average losses of 20% of α-tocopherol and 14% of γ-tocopherol. Analyses carried out on 8 samples of hydrogenated fats gave tocopherol values up to the level of original oils, indicating that the hydrogenation step itself does not destroy the tocopherols. Lastly, 7 samples of mixed fat for margarine production were assayed, and gave values somewhat higher than those calculated from the margarine values, indicating some loss during margarine production.

INTRODUCTION

The dietary intake of tocopherols is intimately connected to the problem of unsaturated fats in nutrition (Horwitt, 1962). The fatty acid intake of a population can be modified to a certain extent through the fat composition of margarine. Vitamins A and D are added to margarines in many countries, and the question of vitamin E addition is under discussion (Weber, Gloor and Wiss, 1962). The intake of margarine fats is of special importance in Scandinavian countries, partly owing to the lack of salad oils in the regime, and partly to the price difference between butter and margarine (in Norway, 4:1).

The methods of tocopherol determination have advanced greatly in recent years, and several chromatographic methods are available (Koefler *et al.*, 1962). We have applied chromatography on columns of alumina, reversed-phase paper-strip chromatography, and spectrophotometric measurement to the determination of α-tocopherol in fish fats (Lambertsen and Brækkan, 1959) and of tocopherols in nuts (Lambertsen *et al.*, 1962).

METHODS

Sampling and saponification. Margarines were bought in the open market, and oils, hardened fats, and margarine fat stock were obtained from Norwegian refineries and margarine manufacturers. The samples were stored in plastic containers at +5°C, and 5-g portions, for determination, were cut out from the center of the margarine samples. Saponification time was 10–15 min in ethanol (aldehyde free) and KOH, with pyrogallol added for protection. Ethyl ether (peroxide-free) extraction was performed mainly according to the USP-16 vitamin A method, followed by quick vacuum evaporation of the extract.

Column chromatography. Preliminary experiments showed that a 250 × 12-mm column of alumina (Brockmann quality, 10% water added), evenly packed and in strict vertical position, gave the necessary separation. The sample was applied in a small volume of hexane (2 ml), and the development proceeded slowly (1 ml/min) using 25 ml of hexane followed by 5% ethyl ether in hexane. A total eluate of 50 ml normally passed before the α-tocopherol fraction came through. This was collected in 10-ml volumetric flasks, controlled by the Emmerie-Engel reaction performed on drops of eluate. When the α-tocopherol fraction had passed the column, elution was continued with 15% ethyl ether in hexane. The γ-tocopherol fraction should pass out of the column, leaving the vitamin A fraction (as seen in u.v. light) still a few centimetres above the outlet. δ-Tocopherol can be eluted together with vitamin A, but was not determined in the present study.

Paper chromatography. The γ-tocopherol fraction from the alumina column was sufficiently pure for spectrophotometric determination, whereas the

α-tocopherol fraction often needed further purification.

Strips of Whatman no. 3MM paper (300 × 15 mm) dipped in 5% squalene in hexane to within 5 cm from one end were run horizontally. The α-tocopherol fraction was applied in hexane to the non-impregnated part of the paper strip. Ethanol (85%), originally used as a developer, resulted in rather broad spot formation on margarine samples. Studies revealed that the reason could be wax-like components in margarine fats, moving behind α-tocopherol. Changing the developer to isopropanol-water (85:15) checked the influence of the unknown component. Four strips constituted one run. The sample was applied to 3 strips, the fourth was used as a blank. One strip was developed with the Emmerie-Engel reagent, and the area corresponding to α-tocopherol cut out of the 3 other strips. Cyclohexane was used as a solvent, and the solution centrifuged to remove lint. The two α-tocopherol fractions were measured spectrophotometrically against the blank extract.

Spectrophotometry. γ-Tocopherol was measured in ethanol at wavelengths 288, 296, and 306 mμ, and the correction formula was: $E_{corr} = 4.082 \cdot E_{296} - (2.268 \cdot E_{288} + 1.814 \cdot E_{306})$, $E^{1\%}_{1cm(296m\mu)} = 93.5$. α-Tocopherol was measured in cyclohexane at wavelengths 285, 298, and 303 mμ, and the correction formula was: $E_{corr} = 3.831 \cdot E_{298} - (1.064 \cdot E_{285} + 2.767 \cdot E_{303})$, $E^{1\%}_{1cm(298m\mu)} = 88$.

RESULTS AND DISCUSSION

The α- and γ-tocopherol values in Table 1 are for 37 samples of Norwegian margarines, in Table 2 for 18 samples of margarines from other countries, and in Table 3 for some hydrogenated fats and mixed margarine fats.

The reproducibility of the method was of the order of ±5%, and the normal percentage of corrections was 15–25%. Recovery was better than 95%. D-α-Tocopheryl hydrogen succinate (DPI) and γ-tocopherol (DPI) were used as standards.

The Norwegian margarine samples, bought in ordinary food stores in 4 different areas (Oslo, Bergen, Trondheim, and Svolvær), comprise most popular brands. The average of the values in Table 1 is 52 μg α-tocopherol per gram of margarine, with a range from 20 to 90 μg/g. Sixteen percent of the samples gave values of less than 40 μg/g.

The γ-tocopherol values divide the margarines into 3 groups: above 100 μg/g, 25–100 μg/g, and under 25 μg/g. The vegetable margarines make up the first group, whereas the two next groups probably denote margarines with average and low vegetable oil content (original and/or hydrogenated). The average value for all

Table 1. Tocopherols in Norwegian margarines (1962) (α-tocopherol:γ-tocopherol in μg/g margarine).

	Bought in			
Brand	Oslo	Bergen	Trondheim	Svolvær
Vegetable				
A	35:200	50:200	50:250	65:170
A (1963)		70:270		
B	50:180	50:110	40:110	60:120
B (1963)		16:180		
C		40:200		40:200
Normal				
D	50:70	40:65	50:70	80:50
D (1963)		60:60		
E	45:50	45:45	35:10	40:10
E (1963)		75:60		
F		20:40	65:90	75:60
G		55:65		
H		30:30		
I				35:15
J				45:20
Extra salted				
K	55:70			65:70
L		45:30		
M	70:15		80:15	
N		20:25	45:10	90:45

Table 2. Tocopherols in margarines from 4 different European countries (α-tocopherol:γ-tocopherol in μg/g margarine).

Brand	Country	Tocopherol
A	U. K.	100:100
B		60:40
C		80:40
D		80:50
A	Western Germany	90:90
B		160:90
C		150:80
D		150:90
E		120:80
F		10:70
G		110:35
H		65:40
A	Denmark	20:100
B		60:65
A	Sweden	150:150
B		60:60
C		60:90
D		85:200

Table 3. Tocopherols in hydrogenated fats and fat mixes for margarine production (α-tocopherol: γ-tocopherol in μg/g fat).

Sample	Type	Tocopherol
A	Hydrogenated marine oil, m.p. 30/32°C	100 : —[a]
B		70 : —
C		35 : —
D	Hydrogenated marine oil, m.p. 38/40°C	100 : —
E		25 : —
	Hydrogenated soybean oil	100 : 400
	Hydrogenated ground nut oil	150 : 200
A	Fat mix for margarine production	85 : 80
B		70 : 220
C		100 : 40
D		90 : 110
E		90 : 110
F		35 : 35
G		100 : 280

[a] γ-Tocopherol is not found in animal fats.

samples was 89 μg per g margarine.

δ-Tocopherol was not determined quantitatively, but thin-layer chromatography of the unsaponifiable matter of the margarines proved its presence in all margarines of group I and in most of those in group II. The presence of δ-tocopherol probably reflects soybean oil in the margarine.

Storage of 10 of the margarine samples for 7 months at 5°C in plastic containers gave average losses of 20% of the α-tocopherol and 14% of the γ-tocopherol content. No correlation of losses with type of margarine could be arrived at.

The 18 samples of margarine from West Germany, U. K., Sweden, and Denmark were analyzed as a check on the Norwegian margarines, and cannot be regarded as representative of the average in the countries of origin (Table 2). Six of the margarines were marked for dietary use and showed α-tocopherol values above 100 μg/g; average values were 140 μg/g α-tocopherol and 88 μg/g γ-tocopherol. The other 12 margarines gave average values of 64 μg/g α-tocopherol and 79 μg/g γ-tocopherol. These margarines correspond well with the Norwegian samples, with average values 23% higher for α-tocopherol and 11% lower for γ-tocopherol.

Ward (1958), in his study on the tocopherol content of British and American margarines, found α-tocopherol values varying from 6.9 to 55.8 μg/g for 13 different margarines, while dimethyl-tocopherols (including γ-tocopherol) varied between 4.5 and 38.9 μg/g. The monomethyl-tocopherols (including δ-tocopherol) varied between 1.1 and 50.5 μg/g. In American margarines (5 samples) the values were: α-tocopherol, 33–158 μg/g; dimethyl-tocopherols, 130–135 μg/g; and monomethyl-tocopherols, 73–201 μg/g.

Swedish margarines have been analyzed by Hellström and Andersson (1956). In six different brands, analyzed repeatedly during two years, the average total tocopherol content was 171 μg per g. The average α-tocopherol content was 48 μg per g, and the average dimethyltocopherol content 108 μg per g.

Täufel and Serzisko (1961) found a total tocopherol content of 137–315 μg per g in German margarines.

Table 3 gives some tocopherol values from hydrogenated fats and from margarine fats taken before emulsification. These values indicate that the hydrogenation step itself does not destroy the tocopherols. The values for hydrogenated vegetable oils and the highest values for hydrogenated fish or whale oils correspond well with those in the original oils. The great variation in the tocopherol content of the hydrogenated marine oils probably reflects the method of refining. Such steps as alkali treatment and deodorization could easily destroy tocopherols.

The average for the tocopherols in the 7 margarine fats, 81 μg/g α-tocopherol and 125 μg/g γ-tocopherol, are somewhat higher than the corresponding values for the margarine samples (calculated for 80% fat in the margarine). It is therefore possible that some losses of tocopherols occur during margarine production.

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Evaluation of the Biuret and Dye-Binding Methods for Protein Determination in Meats

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SUMMARY

The biuret method of protein estimation was compared with the Kjeldahl method. Highly significant positive correlations with Kjeldahl protein of 0.99, 0.99, 0.98, and 0.99 were obtained for ground beef, pork, chicken breast, and cod, respectively. The high correlations between the two methods and the small standard deviations for the biuret values point out the reliability and the accuracy of the biuret method. The same substances were analyzed by the Orange G dye-binding method with highly significant positive correlations with Kjeldahl protein of 0.90, 0.80, 0.94, and 0.95 for ground beef, pork, chicken breast, and cod, respectively. However, the amount of dye bound per g protein varies with the protein content of the sample, and the precision is poor. Orange G dye binding has possibilities for use in analyzing meat proteins only if the preparations and procedures are carefully standardized and the protein content does not vary more than a few percent. With Amido Black 10B, the amount of dye bound was too strongly dependent upon sample size to justify further investigation of this dye for estimation of the protein content of comminuted meats.

Federal and state meat inspection laws specify a maximum percentage of fat for comminuted beef and pork. One factor that prevents substitution of a specific protein composition requirement for the restriction stated for fat is that the most acceptable method of protein determination, the Kjeldahl method, is complicated and time consuming. The specific object of this study was to investigate the applicability of the biuret reaction and dye binding for quantitative determinations of the proteins of comminuted beef, pork, chicken breast, and cod.

It has been observed that proteins are able, under specified conditions, to complex certain organic dye molecules. Some enlightening work has been done (Fraenkel-Conrat and Cooper, 1944) on the determination of total acidic and basic groups of proteins. Many projects based on that study have investigated the applicability of the dye-binding method for quantitative protein determination of food substances.

Orange G binding has been used to determine quantitatively the protein fractions of wheat flour (Udy, 1954, 1956a) and has been adapted for the estimation of total protein in milk (Ashworth and Seals, 1957; Ashworth *et al.*, 1960; Treece *et al.*, 1959; Udy, 1956b). The method has been tried on meat, fish, bean, and nut meals (Bunyan, 1959). Another application of the protein-Orange G complex formation has been in the realm of the protein quality of heat-treated fish and soybean meals (Moran *et al.*, 1963). Amido Black 10B, an anion-active dye, has been used to determine the protein content of milk (Steinsholt, 1957; Raadsveld, 1959) and various forms of milk, rennet caseins, and cheeses (Johnsen and Hadland, 1959). Buffalo Black and Orange G have been investigated for use in protein estimation of ground pork and ground beef (Pearson *et al.*, 1962). Cochineal red A has been used for protein estimation of various luncheon meats and heated ground beef (Meester and Houtepen, 1962).

The biuret reaction involves the reaction of alkaline cupric ions with the peptide linkage. The method has the advantage over most methods in that the color formed is nearly independent of the nature of the protein. It is used routinely in most biochemical laboratories for the estimation of small amounts of protein. The method has

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been used for protein determinations of fractions of cod muscle (Dyer *et al.*,1950), on rabbit livers (Hirayama, 1954), and on fish muscle (Matsumoto and Kanamitsu, 1955). In these three cases, the procedures were elaborate and time consuming.

MATERIALS AND METHODS

The substances analyzed included raw beef adductor muscle from the Animal Husbandry Department, and raw chicken breast from the Poultry Husbandry Department, University of California at Davis; and raw pork, principally from the loin area, and cod fillets, both from a local market. All samples were ground 4–5 times in a Hobart meat grinder, Model N-50, with varying amounts of fat. The beef was diluted with external beef fat, and the others were diluted with lard. All samples were packaged in polyethylene bags in approximately 110-g lots, frozen immediately, and thawed at refrigerator temperature (3°C) about 24 hr prior to analyzing.

Used as standards were macro-Kjeldahl values, determined by the official method of analysis of AOAC (Horwitz, 1955). The samples were digested with sulfuric acid, with cupric sulfate pentahydrate and potassium sulfate as catalysts. The ammonia was trapped in 4% boric acid and titrated with standard acid. Percent nitrogen was converted to percent protein with the factor 6.25.

For moisture determinations, samples of 7.5–9.0 g were dried for 17–20 hr in aluminum pans in a vacuum oven (23–26 inches Hg) at 65–70°C. The loss in weight was considered to be moisture loss.

Orange G, obtained from the National Aniline Division, Allied Chemical Corporation, was labeled as having a 92% total dye content. The stock solution contained 1 mg Orange G per ml in a 0.1*M* citrate-0.2*M* phosphate buffer, pH 2.2. A 0.9–1.2-g sample of meat was weighed out and added, with the glassine weighing paper, to exactly 100 ml of the stock dye solution in a Monel metal container (capacity 360 ml) fitted to a Waring blender motor. The sample was blended for exactly 1 min, allowed to stand exactly 15 min, blended for an additional 15 sec, and then centrifuged in two tubes for 10 min at 11,640 rpm (16,292 × G) in a Servall Model SS-1 centrifuge. A 5-ml portion, taken from each tube at a point below the fat layer and above the protein-dye complex precipitate, was filtered through Whatman No. 1 filter paper to remove the obvious fat particles, which could not be avoided in pipetting. A 1-ml portion of the filtrate was diluted to 100 ml with buffer, and absorbance was read at 475 mμ on a Bausch and Lomb Spectronic 20 colorimeter. The average of four absorbance readings on each sample was used for determining the concentration of unbound dye. The amount of dye added was determined on 100 ml of stock dye solution carried through the same procedure as the sample.

Amido Black 10B dye was from Merck and Company in Germany. The stock solution contained 2 mg dye per ml in a 0.1*M* citrate-0.2*M* phosphate buffer, pH 2.2. The preliminary work with this dye was done under the same conditions as for Orange G. However it was noticed that particles of the Amido Black 10B adsorbed strongly to the weighing paper and to the inside walls of any glass container used. A less concentrated dye solution was unsatisfactory.

The biuret reagent was prepared as described by Layne (1957). A meat sample of 0.9–1.2 g was weighed accurately, and transferred, with the glassine weighing paper, to a 50-ml Ehrlenmeyer flask containing 20 ml of 0.5*M* sodium hydroxide. The sample was partially dispersed with a stirring rod, heated in a boiling-water bath for exactly 10 min, and cooled in an ice-water bath. The contents of the flask were quantitatively transferred to a 50-ml volumetric flask and made to volume with deionized water. The solution was filtered through Whatman No. 3 filter paper to remove the elastin particles and some of the fat. A 15-ml portion of the filtrate was transferred to a polyethylene centrifuge tube, and 15–18 ml of anhydrous ether were added. The tube was capped, and the contents were shaken cautiously but well, before centrifugation at 5820 rpm (8146 × G). Sampling was done from the lower, aqueous layer in the amounts of 0.4, 0.8, and 1.0 ml. Amounts were made up to 1.0-ml quantities, where necessary, with deionized water. Exactly 4.0 ml of biuret reagent were added and mixed, and the optical density of the reaction mixture was read exactly 30 min later at 540 mμ on a Bausch and Lomb Spectronic 20 colorimeter. The blank, which contained 1.0 ml water in place of sample, was prepared in the same way. Crystalline bovine serum albumin (Armour Laboratories) was used for preparation of the standard curve. $E^{1mg}_{540\ m\mu}$ was found to be 0.060 mg^{-1}

RESULTS AND DISCUSSION

Biuret method. Table 1 shows the effect of heating time on the solubilization of the proteins by 0.5*M* NaOH. The time must be long enough to solubilize the proteins but short enough that detectable hydrolysis of the proteins does not occur. Ten minutes was the digestion time used for beef, pork, and chicken breast because in each case

Table 1. Effect of heating time on extraction of beef, pork, chicken breast, and cod proteins.

Species	Time (sec)	Protein (%)
Beef [a]	600	23.2±0.0
	1200	22.4±0.1
	1800	22.1±0.0
	2400	22.0±0.1
	3000	21.7±0.1
Chicken breast [b]	600	24.6±0.3
	700	25.6±0.4
	800	24.5±0.1
Cod-1 [c]	300	16.9±0.3
	600	16.6±0.1
Cod-2 [d]	300	13.0±0.3
	600	13.0±0.1
Cod-3 [e]	300	10.1±0.4
	600	10.3±0.1
Pork [f]	300	17.4±0.3
	600	16.4±0.1
	900	16.8±0.3

[a] Ground beef adductor.
[b] Ground chicken breast.
[c] Cod fillet ground with lard in a ratio of 6:1.
[d] Cod fillet ground with lard in a ratio of 2:1.
[e] Cod fillet ground with lard in a ratio of 1:1.
[f] Ground pork with lard in a ratio of 6:1.

particles were still to be seen up to this time. In all cases of cod analysis, the particles were absent by the end of a 5-min digestion period.

Preliminary work was done with beef in which the extractant was 1.0*M* KCl–0.5*M* NaOH, but this extractant was not as effective as 0.5*M* NaOH alone.

In the development of the method, some values higher than expected were obtained; these were attributed to turbidity due to presence of fat. A similar problem has been overcome by using carbon tetrachloride, chloroform, and diethyl ether (Hirayama, 1954; Williams, 1961). Several alternative methods were investigated here: small aliquots were centrifuged with biuret reagent and anhydrous ether for a total reaction time of 30 min; some were filtered through Whatman No. 2 filter paper; some through Whatman No. 3 filter paper; some were refrigerated; and some larger aliquots were shaken and centrifuged with anhydrous ether before being sampled. The last method gave the best results.

The relationship between percent protein determined by the biuret and by the Kjeldahl methods is shown in Fig. 1 and Table 2. The correlation coefficients for the relationship between percent protein as determined by the biuret technique and Kjeldahl method on beef, pork, chicken breast, and cod are respectively +0.99, +0.99, +0.98, and +0.99. In no case does the line pass through the origin, although the y-intercepts are all quite close to zero. It was expected that the biuret values would, in general, be less than those given by the Kjeldahl method, since the latter technique is believed to account for total nitrogen whereas the biuret reagent theoretically reacts with molecules containing at least two peptide linkages (polypeptides and proteins). It was also felt that the biuret technique employed here did not account for all the elastin content, since it was not completely solubilized by the NaOH. The elastin contents of beef longissimus dorsi and semitendinosus are respectively 0.7 and 2.0% (Doty and Pierce, 1961). There were visible white particles, which must have been elastin. The difference between biuret and Kjeldahl values would be expected to be equivalent to the total of elastin and nonprotein nitrogen content, which is quite low in meats.

Orange G dye binding. The dye binding capacity (DBC) of proteins is defined as the mg of dye bound per g of protein (N × conversion factor 6.25 for meat). Although it

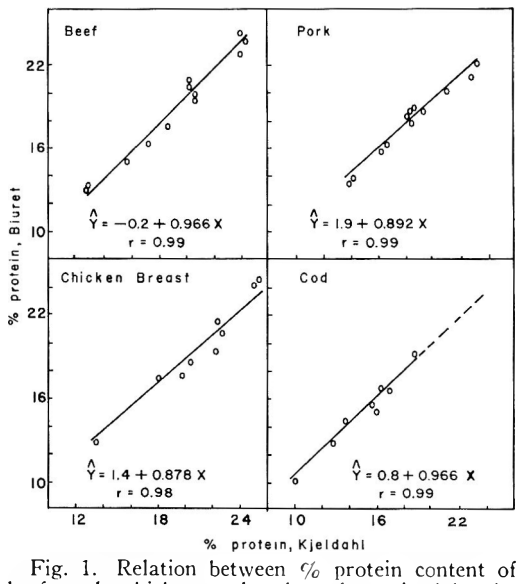

Fig. 1. Relation between % protein content of beef, pork, chicken, and cod as determined by the biuret and Kjeldahl methods. *Y* is percent biuret protein and *X* is percent Kjeldahl protein.

Table 2. Percentage protein and moisture for beef, pork, chicken breast, and cod samples.

Sample and No.	Protein		Moisture (%)
	Kjeldahl (%)	Biuret (%)	
Beef, 10 samples			
Average	19.7 ± 0.3	19.4 ± 0.3	60.29 ± 0.11
Range	24.2 to 12.8	24.3 to 13.0	72.12 to 38.02
Pork, 11 samples			
Average	18.3 ± 0.3	18.1 ± 0.3	55.20 ± 0.18
Range	23.1 to 13.9	22.4 to 13.8	66.58 to 44.78
Chicken breast, 9 samples			
Average	20.9 ± 0.2	20.0 ± 0.3	58.82 ± 0.13
Range	25.2 to 13.4	24.6 to 13.0	71.92 to 35.48
Cod, 8 samples			
Average	14.9 ± 0.2	15.2 ± 0.2	62.49 ± 0.07
Range	18.8 to 9.8	19.2 to 10.2	79.50 to 37.52

is often assumed that each unit weight of protein binds and precipitates a constant amount of dye, through a stoichiometric reaction between the basic amino groups on the protein and acidic groups of the dye, some workers are convinced that the DBC of proteins at a certain pH is not constant, but depends on the concentration of both protein and dyestuff (Ashworth and Chaudry, 1962; Meester and Houtepen, 1962). The amount of orange G dye bound, in mg per g of Kjeldahl protein, has been plotted against % Kjeldahl protein and is presented in Fig. 2. In all cases there is evidence of decrease in DBC with increase in protein content. Possible explanations can be suggested for the trend of the curves. There may be differences in the proportions of proteins in any natural product. The reaction between dye and protein probably involves a series of multiple equilibria that are affected by both dye and protein concentrations. There is also a possibility that excess dye is bound at the lower protein concentrations by nonionic adsorption.

The relationship between mg Orange G bound per g of sample and % Kjeldahl protein is shown in Fig. 3. The t value for pork is 4.425, showing significance at the 1% level. In all cases the equations of the regression lines can be used to calculate % of protein when the X value is known for any given sample.

The y-intercepts for beef and pork are

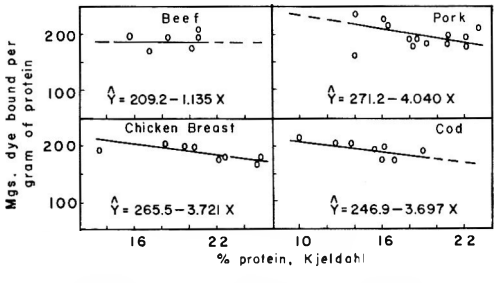

Fig. 2. Orange G dye-binding capacity of beef, pork, chicken, and cod proteins at pH 2.2 as a function of % protein (Kjeldahl). Y is mg dye bound per g of protein and X is percent Kjeldahl protein.

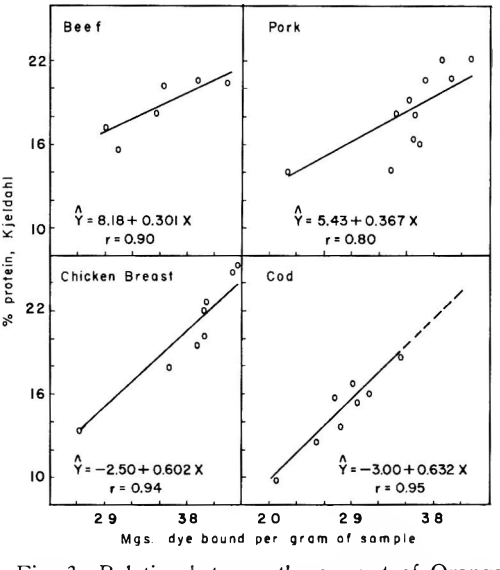

Fig. 3. Relation between the amount of Orange G bound per g sample at pH 2.2 and % protein (Kjeldahl) for beef, pork, chicken, and cod. Y is percent Kjeldahl protein and X is mg dye bound per g of sample.

quite high, considering that the lowest percentage protein contents used were respectively 15.6 and 13.9. There is a strong indication here that as the concentration of protein decreases, the quantity of Orange G bound per unit of sample decreases. This is an important factor in empirical studies, but could probably be corrected for in a practical application of the technique to routine analyses, where the protein concentrations are expected to vary only within 2–4%. A possible explanation of the occurrence is the physical blocking of the formation of the dye-protein complex by the fat molecules present in the samples of lower protein content. The y-intercepts for chicken breast and for cod are much closer to the origin but are on the opposite side of zero, implying that more Orange G is bound per unit of protein in the samples of lower protein content of these species. This occurrence may be due to the presence of excess dye in solution and may represent a physical adsorption of dye molecules onto the protein; or it may represent a problem of steric hindrance in the upper range of protein concentrations due to the bound dye molecules themselves.

Amido Black 10B binding. The results of studies made with Amido Black 10B in which various sample weights were used are presented in Figs. 4 and 5. Both values of r are highly significant. There is a definite trend toward the binding of less dye per g of sample as the sample size increases (Fig. 4). This indicates that there is insufficient dye to react with all the positive

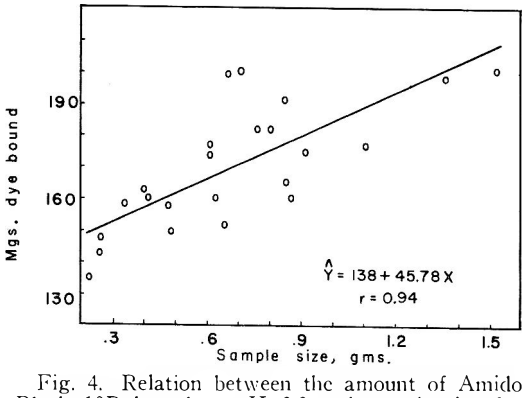

Fig. 4. Relation between the amount of Amido Black 10B bound at pH 2.2 and sample size for beef. Y is mg dye bound and X is sample size in g.

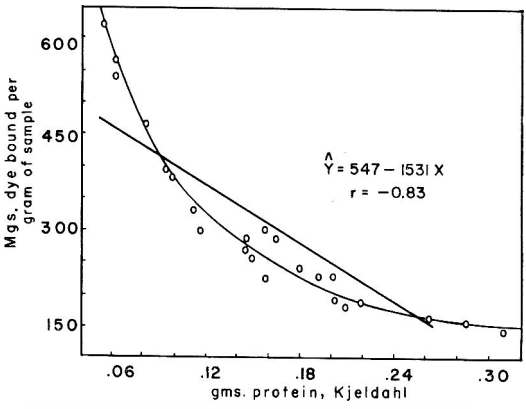

Fig. 5. Relation between the amount of Amido Black 10B bound per g sample at pH 2.2 and protein content (Kjeldahl) for beef. Y is mg dye bound per g of sample and X is g protein (Kjeldahl).

charges of the proteins. The concentration of dye cannot be increased further, because of the low solubility of Amido Black 10B. Only by decreasing the sample size sufficiently (it would have to be below 0.3 g) could we hope to obtain a linear response between sample size and amount of dye bound. The relation between mg dye bound per g sample and g protein (Fig. 5) may be expressed better by means of a curved line, indicating an exponential relation between protein content and amount of dye bound. Since the dye binding was so dependent on sample size, the use of this dye was abandoned.

Cooked beef. Two samples were heat denatured and analyzed for protein by the biuret, Kjeldahl, and Orange G dye-binding methods. The values for the raw and cooked meats were respectively 20.6 and 22.8% by Kjeldahl, 19.9 and 23.3% by biuret, and 40.95 and 45.21 mg dye bound/g by Orange G. The increase in % protein is due to moisture loss on cooking. The biuret and dye-binding methods, when compared with the Kjeldahl method, gave as good results on cooked meat as on raw meat.

Comparisons. Excellent correlation was obtained between the percent protein found by the biuret and by the Kjeldahl methods (Table 2 and Fig. 1). The standard deviations of the data for the biuret method are also shown to be as good as those for the Kjeldahl method (Table 2), indicating the reliability and precision of the biuret method.

The advantages of the biuret method are realized in a saving of time for analyses, in the fact that the analyses can be performed in readily available laboratory equipment, and in the fact that the solutions required are much simpler than those needed for the Kjeldahl method. The biuret method could readily be automated by further refinement of equipment and method.

Although the r values for the curves in Fig. 3 are significant at the 1% level, the poor precision of the data offers a reason to be skeptical of the Orange G dye-binding method. However, it is reasonable to believe that the Orange G dye-binding method could be satisfactorily adapted to routine estimations of the protein content of meats where the range of values does not exceed 2–4%. The Orange G dye-binding method could easily be automated and would offer a much less time-consuming technique for protein analyses than the Kjeldahl method. However, the repeatability of results by the dye-binding method is not good enough to warrant the substitution of this method for the more complicated yet more reliable Kjeldahl method.

Since, at the dye concentration necessary for meat analyses, the amount of Amido Black 10B dye bound is strongly dependent upon sample size, and since the relation between dye bound per g sample and the g protein (Kjeldahl) in the sample is complex, this dye is apparently not satisfactory for estimation of the protein content of comminuted meats.

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Processing Characteristics of Porcine Muscle Related to pH and Temperature During Rigor Mortis Development and to Gross Morphology 24 hr Post-Mortem[a, b, c]

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SUMMARY

pH and temperature in muscle during the onset of rigor mortis as well as gross morphology of muscle 24 hr post-mortem were related to fluid losses and associated properties during refrigerated storage, cooking, and thawing. When rigor mortis onset occurred at pH values below 5.9 and temperature above 35°C, the longissimus dorsi muscle became pale and exudative. Evaporative cooking losses amounted to 40–45% of the sample weight and resulted in slow cooking rates and high shear-force values. Conversely, when the onset of rigor mortis occurred while pH values remained above pH 6.0 the muscles were dark and firm, with superior juice-retaining properties. Less than 20% of the sample weight was lost by evaporation during cooking, allowing the muscle temperature to rise at about twice the rate found for pale exudative muscle. Some muscles were found to he exudative and have low pH values (5.2) while retaining a dark red color. These muscles were found to have storage, cooking, and organoleptic properties similar to those found for pale exudative muscle. Storage of muscle samples at either 0°C or 6°C did not improve color, pH, juice retention or cooking characteristics. Gross morphology of muscle chilled 24 hr was associated more closely with thaw drip formation than with freezing or thawing conditions.

INTRODUCTION

The rate of glycolysis (Briskey and Wismer-Pedersen, 1961; Sayre *et al.*, 1963a) and physiological conditions during rigor mortis influence the post-rigor properties of muscle tissue. With rapid glycolysis and onset of rigor mortis at a pH below 5.7 and temperature above 35°C, the muscle becomes extremely pale, soft and exudative and shows a marked loss in sarcoplasmic and myofibrillar protein solubility (Sayre and Briskey, 1963). These muscles have extremely poor fluid retention properties (Briskey *et al.*, 1960; Sayre *et al.*, 1963a,b) and have been shown to lose excessive weight during processing (Carpenter, 1962; Karmas and Thompson, 1964), although reports on organoleptic properties have been conflicting (Judge *et al.*, 1960; Lewis *et al.*, 1962).

This investigation was conducted to relate pH and temperature conditions during rigor mortis onset and gross morphology of chilled muscle 24 hr post-mortem to fluid retention and associated properties during cooking, storage, and thawing.

EXPERIMENTAL

Phase I. In the first phase of this study, the muscles were grouped according to pH and tem-

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perature at rigor mortis onset. The time course of rigor mortis was determined with a "rigorometer" apparatus described by Briskey *et al.* (1962). pH values were determined by placing a combination probe electrode directly on the freshly cut cross-sectional surface of the muscle fibers. Samples for sarcoplasmic and myofibrillar protein determinations were obtained at death, at onset and completion of rigor mortis, and at 24 hr post-mortem (24 hr) as described by Sayre and Briskey (1963). Muscle samples (24 hr) were subjectively scored for gross morphology on a 4-point scale (Sayre *et al.*, 1963a): from 4, dark firm muscle to 1, pale exudative muscle. Reflectance of 485-mμ light was used as an objective measurement of color intensity. The reflectance was measured with a Bausch and Lomb "Spectronic 20" spectrophotometer with reflectance attachment and was expressed as a percentage of the reflectance from a magnesium carbonate block. The juice-retaining properties of 24-hr samples were determined according to the filter-paper absorption method of Grau and Hamm (1953) as modified by Urbin *et al.* (1962) and expressed as the ratio of total area to meat film area. Consequently, a larger ratio indicated a more exudative condition of the muscle.

Cooking characteristics were determined with sections of longissimus dorsi muscle (24 hr.). External fat and surface connective tissue were removed, and samples were cut to a standard size of 1.25 ×2.5 × 5 in. to provide a uniform surface area. The individual sections were roasted on an open rack in an institutional oven at 177°C to an internal temperature of 85°C. Rate of temperature increase was determined with centrally located thermocouples and was continuously recorded. Evaporation loss represented the difference between initial and final weight of the sample and container. Shear force was determined with a Warner-Bratzler shear press on three ½-in. cores taken parallel to the muscle fibers.

Phase II. The second phase of this study concerned cooking, storage, and thawing characteristics of muscles representing various gross morphological categories 24 hr post-mortem (Briskey, 1963). These categories were selected as representative of various post-mortem glycolytic conditions and rates of rigor mortis development. The three categories were: 1) pale exudative, representing rapid glycolysis, with the onset of rigor mortis occurring at a high muscle temperature and low pH; 2) dark exudative, representing extensive glycolysis occurring at a moderate rate and thereby achieving a low (approx. 5.2) 24-hr pH, with rigor mortis onset taking place at a low pH and moderate to low muscle temperature; 3) dark firm, representing a slow and limited glycolysis, resulting in onset of rigor mortis at high muscle pH. Six longissimus dorsi muscles were obtained to represent each category as the carcasses were cut at 24 hr post-mortem. The longissimus dorsi muscles were excised, freed of all external fat and surface connective tissue, and used for subsequent determination of physical properties and cooking qualities before and after 8 days at either 0 or 6°C. Color, pH, and juice retention were determined as previously described in Phase I. Moisture, fat, and protein were determined as previously described by Sayre *et al.* (1963a). Weight losses during the 8-day holding period were measured on uniformly prepared samples (1.25 × 2.5 × 5.0 in.) held in a moisture-saturated atmosphere at 0 and 6°C. Protein determinations were conducted on representative samples of accumulated exudate. Similar sections were cooked as described in Phase I, before and after holding, and evaluated by an eight-member trained organoleptic panel.

Portions of four additional loins representing the pale exudative and the dark firm categories were frozen at −29 and −78°C and subsequently thawed at 1, 7, and 20°C. Thaw drip, cooking rate, cooking evaporation and shear force were measured as previously described in Phase I.

RESULTS AND DISCUSSION

Phase I. Muscles were assigned to six different groups, depending upon pH and temperature at rigor mortis onset. The data from this investigation are tabulated in Table 1. The pH of muscles in group A and B fell to 5.6 or below at onset of rigor mortis; however, muscle temperature remained above 35°C in group A and below 35°C in group B. Muscles in groups C and D were at an intermediate pH, between 5.7 and 5.9, at rigor mortis onset, with the muscle temperature above 35°C in group C and below 35°C in group D. Onset of rigor mortis occurred at relatively high pH values of 6.0 or above in groups E and F. The short delay phase of muscle in group E resulted in onset temperatures above 35°C at onset of rigor mortis, whereas muscle temperatures in group F fell below 35°C prior to onset of rigor mortis. Muscles with onset of rigor mortis occurring at medium to low pH values (<5.9) while the temperature remained high became pale and soft, with inferior juice-retaining properties. These muscles (group A and C) subsequently lost 40–46% of their weight by

Table 1. Physical properties of chilled muscles grouped according to pH and temperature at rigor mortis onset.

Group		A	B	C	D	E	F
Muscle conditions at onset of rigor	pH	<5.6	<5.6	5.7–5.9	5.7–5.9	>6.0	>6.0
	Temp.	>35°	<35°	>35°	<35°	>35°	<35°
Subjective score (24 hr)[a]	$\bar{x}$	0.1	2.2	0.5	2.9	3.8	3.2
	s	0.0	0.3	0.3	0.3	0.4	0.3
Expressible-juice ratio (24 hr)[b]	$\bar{x}$	4.0	2.9	2.6	2.6	1.8	2.2
	s	0.2	0.1	0.2	0.2	0.2	0.7
Evaporative cooking loss (%)	$\bar{x}$	40.2	32.1	46.4	26.8	11.2	19.8
	s	2.4	1.2	3.5	1.2	7.3	2.2
Cooking rate (°C/min)	$\bar{x}$	0.8	1.0	0.7	1.1	1.8	1.7
	s	0.1	0.1	0.1	0.1	0.2	0.4
Shear force (lb)	$\bar{x}$	9.3	8.2	9.2	8.8	4.9	4.6
	s	1.5	0.7	1.7	0.6	0.1	1.4

[a] Based on a scale from 1, pale, to 4, dark.
[b] Ratio of total area to meat film area.

evaporation during cooking. Because of the high evaporative loss, the rates of temperature increase in groups A (low pH, high temperature) and C (medium pH, high temperature) were less than one-half the rates of increase in groups E and F (high pH). The association of cooking rate to the conditions of rigor mortis and ultimate appearance may explain some of the differences noted in this characteristic under commercial conditions. Shear-force values were also markedly lower in groups E and F, reflecting a greater tenderness in muscle that had low evaporative losses during cooking. The relation between cooking loss and tenderness gives further support to reports of Baker (1942), Hamm (1953), Leistner (1953), Wierbicki *et al.* (1954), and Hamm and Grau (1958) that cooking loss greatly influenced the juiciness and texture of meat.

Table 2. Simple correlations between some physical properties of post-mortem muscle and cooking characteristics.

	Cooking evaporation	Cooking rate	Shear force
pH			
0 hr	−.54*	.38	−.64**
Onset[a]	−.76**	.73**	−.81**
Completion[a]	−.72**	.69**	−.85**
24 hr	−.59*	.58*	−.63*
Temperature			
0 hr	.69**	−.62*	.50
Onset	.22	−.13	−.07
Completion	.14	−.04	−.13
24 hr	.24	−.31	.23
Sarcoplasmic-protein solubility (24 hr)	−.78**	.72**	−.53*
Myofibrillar-protein solubility (24 hr)	−.76**	.60*	−.45
Subjective color score (24 hr)[b]	−.91**	.80**	−.60*
Expressible-juice ratio (24 hr)[c]	.75**	−.77**	.57*
Cooking evaporation	....	−.90**	.73**
Cooking rate	....	....	−.73**

[a] Phase of rigor mortis (Briskey *et al.*, 1962).
[b] Based on a scale from 1, pale, to 4, dark.
[c] Ratio of total area to meat film area.

Table 2 contains the correlation coefficients between many of the physical and physiological factors observed in this study. The pH at onset and completion of rigor mortis was highly correlated ($P<.01$) with the evaporative cooking loss, cooking rate, and tenderness of the muscle. Muscle temperatures at death were correlated significantly with cooking evaporation and cooking rate, but subsequent muscle temperatures did not have a close association with those factors. The solubility of sarcoplasmic and myofibrillar proteins (24 hr) was highly associated ($P<.01$) with cooking evaporation. Sayre and Briskey (1963) showed that conditions of acidity and temperature at rigor onset determine to a large extent the protein solubility and juice-retaining properties of muscle at 24 hr post-mortem. These correlations give further clarity to the fact that protein solubility and factors affecting it greatly influence processing losses and contribute to product variability. The relationships of tenderness with these factors are in direct support of work on poultry muscle by de Fremery and Pool (1963). Subjective color score (24 hr) and expressible-juice ratio (24 hr) were both significantly ($P<.01$) correlated with cooking evaporation, cooking rate, and ($P<.05$) shear force. A highly significant ($P<.01$) negative correlation existed between cooking evaporation and cooking rate, suggesting that the retardation of increase in muscle temperature resulted from the cooling effect of evaporative cooking losses.

Phase II. The data in Table 3 indicate no appreciable differences in protein and moisture, and only slight differences in percent fat among the three muscle categories. The pH values indicate that glycolysis was only limited in dark dry muscle but was extensive in both types of exudative muscle. The low expressible-juice ratios for the dark firm tissue and the high expressible-juice ratios for the dark exudative tissue can be explained in terms of ultimate pH values (Sair and Cook, 1938; Briskey *et al.*, 1960; Briskey, 1963). Conversely, the high reflectance values and high expressible-juice ratios of the pale exudative tissue are the result of rapid post-mortem glycolysis (Briskey and Wismer-Pedersen, 1961).

Table 4 shows cooking characteristics for

Table 3. Proximate composition and physical properties of three categories of chilled, uncooked porcine longissimus dorsi muscle.

	Dark Firm		Dark Exudative		Pale Exudative	
	$\bar{x}$	$s\bar{x}$	$\bar{x}$	$s\bar{x}$	$\bar{x}$	$s\bar{x}$
Protein (%)	21.3	0.4	20.3	0.4	20.7	0.5
H_2O (%)	72.4	0.1	74.3	0.4	73.7	0.5
Fat (%)	7.1	0.4	5.3	0.5	5.2	0.9
pH	6.1	0.2	5.2	0.1	5.2	0.0
Expressible-juice ratio[a]	1.6	0.2	2.7	0.1	2.9	0.1
Surface reflectance (%)[b]	12.8	0.6	19.5	1.5	23.6	1.3
Subjective color score[c]	3.8	0.1	2.8	0.1	1.6	0.2

[a] Ratio of total area to meat film area.
[b] Expressed as a percentage of the reflectance from magnesium carbonate.
[c] Based on a scale from 1, pale, to 4, dark.

Table 4. Cooking and organoleptic characteristics of three categories of porcine longissimus dorsi muscle.

	Dark Firm		Dark Exudative		Pale Exudative	
	$\bar{x}$	$s\bar{x}$	$\bar{x}$	$s\bar{x}$	$\bar{x}$	$s\bar{x}$
Drip (%)	0.8	0.2	1.9	0.2	1.3	0.2
Evaporation (%)	14.5	1.0	33.1	3.1	33.7	2.2
Cooking rate (°C/min)	1.5	0.0	1.1	0.1	1.0	0.1
Tenderness score[a]	8.1	0.4	4.9	0.5	6.3	0.5
Juiciness score[a]	7.9	0.2	4.4	0.4	4.6	0.5

[a] Based on a 9-point scale with 9 most desirable.

the three muscle categories. Since all external fat was removed and large surface areas were exposed, only small quantities of drip accumulated and essentially all of the cooking loss resulted from evaporation. The magnitude of difference in percent cooking loss between categories was in accord with results from Phase I of this study. These data are in agreement with data of Meyer *et al.* (1963) that showed major differences in cooking losses between muscle differing in juice-retaining properties; however, the reported range for each group was considerably greater than was observed in this study.

The differences in cooking rate also agree with the findings in Phase I; however, the similarity between dark exudative and pale exudative muscles in both evaporation and cooking rate emphasizes the importance of pH to juice retention, as discussed by Hamm (1960). The marked superiority in tenderness and juiciness of dark firm muscle and the similarity in the values for these characteristics between dark exudative and pale exudative products may explain the disagreement between the reports of Judge *et al.* (1960) and Lewis *et al.* (1962) in regard to the organoleptic properties of pale, soft, exudative muscle. Lewis *et al.* (1962) reported that dark muscle of high pH was significantly more tender than pale, watery muscle, which is in agreement with these findings. Conversely, Judge *et al.* (1960) reported that pale, watery muscle was more tender than normal muscle. It is conceivable that the control group in the work of Judge *et al.* (1960) included dark muscles of a watery nature, which might have confounded the cooking results. The finding that juice-retaining properties are related to tenderness is in agreement with work of Wierbicki *et al.* (1956, 1957) and Deatherage (1963).

Table 5 shows how holding for an 8-day period affects certain properties of the three muscle categories. Exudative muscle lost up to 10% of its weight, compared with 1–3% loss from dark firm muscle, and the accumulated drip contained 11–14% protein. Drip loss from all muscle groups was greater when held at 6° than at 0°C. No improvements in pH, expressible juice, color, or cooking characteristics resulted from holding at either 0 or 6°C.

Table 6 shows the influence of freezing and thawing on pale exudative and dark firm muscle. Although there was slightly

Table 5. Characteristics of three categories of porcine longissimus dorsi muscle after eight days' storage.

Characteristics	Storage temp. (8 days)	Dark Firm $\bar{x}$	Dark Firm $s\bar{x}$	Exudative $\bar{x}$	Exudative $s\bar{x}$	Pale $\bar{x}$	Exudative $s\bar{x}$
Storage drip (%)	0°C	1.2	0.6	6.9	0.8	8.2	0.7
	6°C	3.2	0.4	9.4	0.6	10.3	1.1
Expressible-juice ratio[a]	0°C	1.5	0.1	3.2	0.2	3.4	0.1
	6°C	1.7	0.1	3.4	0.5	3.5	0.1
pH	0°C	6.3	0.2	5.3	0.1	5.2	0.0
	6°C	6.3	0.3	5.3	0.0	5.2	0.0
Surface reflectance (%)[b]	0°C	14.3	2.8	17.3	2.2	23.1	0.3
	6°C	13.2	2.2	15.8	3.8	25.0	0.8
Cooking drip (%)	0°C	0.7	0.1	1.1	0.0	1.1	0.2
	6°C	1.3	0.2	1.3	0.0	1.5	0.2
Cooking evaporation (%)	0°C	15.2	1.6	36.7	1.8	34.3	1.8
	6°C	13.0	0.5	34.5	3.2	36.6	1.6
Cooking rate (°C/min)	0°C	1.8	0.1	0.9	0.1	1.1	0.1
	6°C	1.8	0.1	1.2	0.1	1.1	0.2

[a] Ratio of total area to meat film area.
[b] Expressed as a percentage of the reflectance from magnesium carbonate.

Table 6. Effect of freezing and thawing temperature on two categories of porcine longissimus dorsi muscle.

	Dark firm						Pale exudative					
Freezing temp. (°C)	−29°			−78°			−29°			−78°		
Thawing temp. (°C)	1°	7°	20°	1°	7°	20°	1°	7°	20°	1°	7°	20°
Thaw drip (%)	4.5	3.4	6.6	1.1	2.3	2.8	10.8	9.7	15.3	9.8	10.5	14.7
Cooking evaporation (%)	10.4	12.4	14.8	23.2	12.8	11.9	30.8	32.7	33.7	34.9	36.2	35.4
Cooking rate (°C/min)	1.9	2.3	2.0	2.3	2.2	2.3	1.3	1.9	1.8	1.7	1.8	1.8
Shear force (lb)	5.8	6.7	4.8	6.0	6.3	5.1	8.2	7.1	7.3	9.0	8.8	8.7

less thaw drip loss from the dark firm muscle when it was frozen at −78°C, there was no difference in drip accumulation from pale exudative muscle regardless of freezing rate. No significant differences were noted in quantity of drip from muscles thawed at 1 and 7°C; however, thawing at 20°C caused greater drip loss from both muscle categories. Freezing or thawing temperatures did not affect evaporative cooking loss or rate of temperature increase. Shear-force values of frozen and thawed muscles were consistently lower for dark firm muscle than for pale exudative muscle, but were not influenced by freezing and thawing temperatures. Drip loss, shear values, cooking evaporation, and cooking rate appeared to be more highly dependent upon the gross morphological condition of the muscle prior to freezing than upon freezing and thawing conditions.

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Meat Curing
The Action of NaCl on Meat Electrolyte Binding

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SUMMARY

Blended mixtures of meat and water containing different concentrations of NaCl were stored for 24 hr at 3°C and further stored for 1 hr at either 3 or 70°C. Then water, nitrogen, chloride, pH, sodium, potassium, calcium, magnesium, and zinc were determined in aqueous extracts obtained by centrifugation. The results show that little or no sodium, potassium, or chloride was bound at 3 and 70°C. At 3°C, the addition of NaCl resulted in an increase of free calcium, magnesium, and, to a lesser extent, zinc. On heating at 70°C, with no added NaCl, all of the magnesium was free, soluble calcium increased, and zinc decreased. At 70°C, on addition of NaCl, free calcium increased and zinc decreased. Zinc was the only electrolyte that was substantially and strongly associated or bound with soluble protein.

INTRODUCTION

Cured meat and meat products in excess of 7 billion pounds are produced annually in this country. Improvements are constantly sought to improve both quality of products and efficiency of production. The changes in curing methods that have recently evolved have been aimed chiefly at reducing curing time and streamlining production methods. Further development of process modifications and improvements can be greatly aided by a more accurate and comprehensive knowledge of the mechanisms of curing.

The effects of added electrolytes have been stressed in previous investigations of curing. Also prominent among the subjects investigated have been color development and stability, penetration of curing agents, and, particularly, water retention. Evidence has also been obtained indicating that natural meat electrolytes, particularly Ca and Mg, have a role in determining the properties of cured meats (Wierbicki *et al.*, 1957a,b). This role is not clear, owing in part to a lack of agreement among investigators, but mainly to the fact that investigation has been relatively limited in amount. Existing information on the reorganization of natural meat electrolytes after the death of animals, during and after rigor (Hamm, 1958, 1959; Arnold *et al.*, 1956), and the interactions that follow the addition of curing agents, is valuable but not adequate in any of its phases.

The present investigation deals with determining the extent of the movement and binding of natural meat electrolytes following the addition of NaCl, the principal curing agent. In this, blended mixtures of meat and water containing increasing concentrations of NaCl were produced, stored 24 hr at 3°C, and then further stored 1 hr at either 3 or 70°C. Then water, nitrogen, chloride, pH, sodium, potassium, calcium, magnesium, and zinc were determined in centrifugally obtained aqueous extracts. The purpose was to determine whether electrolytes became redistributed between the soluble phases of blended meat and water mixtures at 3°C with the addition of NaCl, whether this distribution was affected by increasing concentrations of NaCl, and the changes that occur on heating at 70°C. To differentiate the quantities of free and protein-bound electrolytes in the aqueous phase, fractionation of free and protein-bound electrolytes was accomplished by ultracentrifugation.

EXPERIMENTAL

Preparation of samples. Rounds of eight steers were obtained 6–8 days after slaughter. Samples from the muscles of three were used in determining free electrolytes in aqueous phases as affected by treatments with NaCl and heat (Tables 1, 2, 3, 4; Figs. 1, and 2); those from two others were used in investigating protein sedimentation by ultracentrifugation (animals A and B, Table 5); and those from three were used in determining the amounts of ions bound by proteins (Tables 6, 7, and 8). The semimembranosus and semitendenosus muscles were removed and trimmed to remove separable fat and connective tissue, and portions were cut into cubes. The cubes were ground twice through an electric food chopper, and the ground meat was mixed thoroughly. Slurries were prepared by mixing meat 1:2 with ice-cold water (w/w) to which solid NaCl was added to produce concentrations of 0.97, 1.9, and 3.7% NaCl in the water present (added water plus the water content of the meat). The actual NaCl concentrations obtained as determined by measurements of sodium and chloride content varied slightly from the latter figures because of small differences in the moisture content of the meat. The slurries were then homogenized in a Servall Omnimixer, the cup being immersed in an ice bath. (Trade names are mentioned for identification, implying no endorsement.) The machine was operated for four 15-second intervals interspaced with 15-second cooling periods, which avoided noticeable temperature rise. The homogenates were then stored for 24 hr at 3°C.

Preparation of supernatant fractions. Aqueous extracts were prepared from portions of the homogenates after storage at 3°C, whereas others were prepared after heating the homogenates at 70°C. To prepare the former, portions of homogenates that had been stored at 3°C were centrifuged in a Servall centrifuge with head SS-1 for 60 minutes at 20,000 rpm (48,200 × G). Homogenates to be heated were transferred to centrifuge bottles and heated 1 hr at 70°C in a water bath. The heated homogenates were centrifuged with head SS-2 for 30 min at 10,000 rpm (16,300 × G), and the supernatant fraction recentrifuged with head SS-1 for 30 min at 20,000 rpm (48,200 × G). The supernatant fractions obtained were decanted and stored at 3°C for subsequent analysis and ultracentrifugation.

Preparation of protein-free supernatant fractions. Supernatant fractions from homogenates containing 0.0 and 3.7% added NaCl, representing both 3 and 70° treatments, were ultracentrifuged under refrigeration in a Spinco Model L fitted with head No. 40 for 24–32 hours at 40,000 rpm (144,000 × G). The top third portion of clear liquid was removed and stored at 3°C until analyzed.

Analytical determinations. Samples of meat and aliquots of supernatant fractions were ashed by a procedure described previously (Berman, 1960). Stock solutions were prepared from these ashed samples, and suitable aliquots were used for the determination of sodium, potassium, calcium, magnesium, and zinc. Sodium and potassium were determined by flame photometry, corrections being made for the presence of each other (Beckman Manual, 1954). Calcium was directly determined by EDTA titration (Patton and Reeder, 1956); magnesium was determined as the magnesium butyl amine hydroxyquinoline complex in chloroform (Umland and Hoffman, 1957); and zinc was determined as the dithizonate in carbon tetrachloride (Sandell, 1950). Chloride, total nitrogen, non-protein nitrogen, and water content and pH values were determined on separate aliquots of the extracts. Chloride was determined with a modified Volhard method (Caldwell and Moyer, 1935), and total nitrogen as described by the Official Methods of Analysis (AOAC, 1955). Moisture content of both residues and supernatant fractions was determined by the oven-drying method (Windham, 1953). Non-protein nitrogen was determined by the micro-Kjeldahl method on clear filtrates obtained after precipitation of the proteins with tungstic acid (Folin and Wu, 1919). A Beckman Model G pH meter was used to determine pH values. The ultracentrifugal sedimentation patterns were determined with a Spinco Model E ultracentrifuge at 20°C and 59,780 rpm.

RESULTS

Composition of meat samples. Table 1 shows the moisture, nitrogen, and electrolyte content of the meat samples.

pH values and nitrogen and water content. Table 2 shows the pH values and the nitrogen and water content of the supernatant fractions from the unheated and heated homogenates. At 3°C, increasing the concentration of NaCl decreased moisture content, increased nitrogen content, and had little effect on the pH values of the supernatant fractions. At 70°C, with increasing concentration of NaCl, moisture content decreased and nitrogen content increased. A small increase in pH was produced by addition of NaCl. Heating to 70°C increased pH and water content, and decreased the nitrogen content of the supernatant fractions.

Electrolyte content of supernatant fractions. Tables 3 and 4 show the electrolyte content of the extracts of unheated and heated homogenates. At 3°C, increasing concentrations of NaCl increased sodium, chloride, calcium, magnesium, and

Table 1. Average (3 animals) composition of meat samples (mg/g meat).

Moisture	Nitrogen	Cl	Na	K	Ca	Mg	Zn
739	34.85	0.380	0.394	4.10	.0323	0.218	.0267

Table 2. Average (3 animals) pH values, nitrogen, and water content of supernatant fractions of heated and unheated homogenates.

	pH				Water content				Nitrogen content			
	% NaCl				% NaCl				% NaCl			
Treatment	3.7	1.9	0.97	0.0	3.7	1.9	0.97	0.0	3.7	1.9	0.97	0.0
3°C	5.50	5.52	5.50	5.51	.930	.950	.960	.973	5.38	4.59	4.41	3.95
Heated at 70°C	5.75	5.76	5.79	5.70	.948	.964	.974	.984	1.99	1.90	1.85	1.80

Table 3. Average (3 animals) electrolyte content of the supernatant fractions of homogenates treated at 3°C.

Cl mg/g H_2O				Na mg/g H_2O				K mg/g H_2O			
% NaCl				% NaCl				% NaCl			
3.7	1.9	0.97	0.0	3.7	1.9	0.97	0.0	3.7	1.39	0.97	0.0
22.3	11.3	5.70	0.126	15.0	7.61	4.01	0.140	1.40	1.44	1.49	1.49

Ca (mg/g H_2O) × 10^3				Mg (mg/g H_2O) × 10^3				Zn (mg/g H_2O) × 10^3			
% NaCl				% NaCl				% NaCl			
3.7	1.9	0.97	0.0	3.7	1.9	0.97	0.0	3.7	1.39	0.97	0.0
9.47	7.51	5.89	5.50	75.9	72.1	70.4	53.1	5.15	5.14	5.18	4.41

Table 4. Average (3 animals) electrolyte content of the supernatant fractions of homogenates heated at 70°C.

Cl mg/g H_2O				Na mg/g H_2O				K mg/g H_2O			
% NaCl				% NaCl				% NaCl			
3.7	1.9	0.97	0.0	3.7	1.9	0.97	0.0	3.7	1.9	0.97	0.0
22.2	11.2	5.60	0.123	14.9	7.73	3.96	0.147	1.40	1.45	1.50	1.53

Ca (mg/g H_2O) × 10^3				Mg (mg/g H_2O) × 10^3				Zn (mg/g H_2O) × 10^3			
% NaCl				% NaCl				% NaCl			
3.7	1.9	0.97	0.0	3.7	1.9	0.97	0.0	3.7	1.9	0.97	0.0
9.58	8.90	8.77	9.04	81.8	81.4	81.5	79.3	0.384	0.576	0.644	0.691

zinc content, and slightly decreased potassium content. At 70°C, increasing concentration of NaCl increased sodium, chloride, and calcium content; magnesium content remained constant, while zinc content decreased. Heating at 70°C had little or no effect on the sodium, chloride, and potassium content of the extracts, but increased the calcium and magnesium content and decreased the zinc content.

Percentage of total electrolytes and nitrogen present in the aqueous phase. Figs. 1 and 2 show the amount of each electrolyte extracted, expressed as the percentage of the total of each electrolyte present in the meat. The equation

$$\frac{\text{electrolyte content of supernatant (g/g } H_2O) \times \text{total water (g)}}{\text{electrolyte content of meat (g/100 g meat)}} \times 100$$

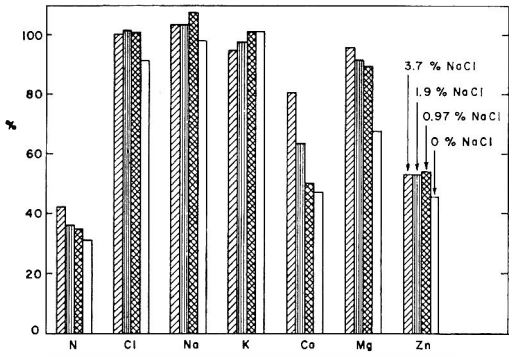

Fig. 1. Average percentages of electrolytes extracted in the aqueous phase of 3°C-treated homogenates.

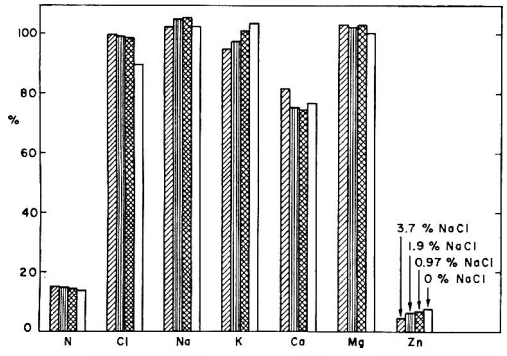

Fig. 2. Average percentages of electrolytes extracted in the aqueous phase of 70°C-treated homogenates.

where total water, g = 200 g + water content of 100 g meat, g was employed in calculating the percentage of electrolyte in the aqueous phase. The assumptions made are that the electrolytes are in equilibrium between the aqueous phase of the supernatant and the aqueous phase retained in the residue. At 3°C, 91.6% of the chloride was soluble in the presence of no added NaCl, while all was soluble in samples containing added NaCl. Sodium and potassium were totally soluble at all concentrations. Increasing NaCl content increased calcium, magnesium, zinc, and nitrogen solubility. Soluble calcium increased from 46.6 to 80.2%; magnesium from 67.2 to 95.8%; zinc from 45.7 to 52.9%; and nitrogen from 31.1 to 42.5%. In homogenates heated at 70°C, sodium, potassium, and magnesium were totally soluble at 0.0% concentration, as they also were on increasing the concentration of NaCl. Chloride was 89.6% soluble in the presence of no added NaCl, and totally soluble at the three levels of added NaCl. Increasing the concentration of NaCl had little effect on the solubility of calcium except for a slight increase at 3.7%. Increasing the concentration of NaCl increased the solubility of nitrogen from 14.1 to 15.7% and decreased the solubility of zinc from 7.7 to 4.5%. Comparison of the amounts of electrolytes soluble in the aqueous fraction of unheated and heated homogenates shows that heating decreased the percentage of soluble nitrogen nearly threefold, had little effect on the solubility of chloride, had no effect on the solubility of sodium and potassium, increased the solubility of calcium at all NaCl levels, produced totally soluble magnesium at all levels, and decreased the solubility of zinc enormously.

Electrolytes and nitrogenous substances in ultracentrifugal supernatant fractions. Table 5 shows the amount of total nitrogen and non-protein nitrogen of a supernatant fraction from a homogenate containing 3.7% added NaCl, before and after ultracentrifugation. As shown in the table, total nitrogen and the non-protein nitrogen contents were practically identical, further showing that a protein-free solution was obtained. Table 6 shows the content of water, nitrogen, chloride, and sodium; and Table 7 shows the content of potassium, calcium, magnesium, and zinc in the supernatant fractions of unheated and heated homogenates before and after ultracentrifugation at 144,000 × G. The content of nitrogen decreased greatly, as expected. Water, chloride, sodium, potassium, calcium, and magnesium decreased to only a small extent. The zinc content decreased greatly. Treatment with NaCl at the 3.7% level, as compared with the 0.0% level, increased the amount of non-protein nitrogen in the supernatant 0.14 mg N/ml H_2O, or 10.9%. The non-protein nitrogen contents of supernatant fractions from treatments with 3.7% NaCl at both 3 and 70°C were similar. Therefore, no fragmentation of protein apparently took place at 70°C. Treatment with 3.7% NaCl at 3°C increased the nitrogen content by 1.59 mg N/ml H_2O; consequently, after correcting for the 0.14 mg N/ml H_2O increase in non-protein nitrogen, 1.45 mg/ml H_2O of salt-soluble protein nitrogen was found to be solubilized by 3.7% NaCl. Comparison of the supernatant fractions from treatment with 3.7% NaCl at 70°C, before and after ultracentrifugation, shows that 0.28 mg N/ml H_2O of protein was sedimented. This amount, representing 18.5% of the salt-soluble protein, was not heat-coagulated at 70°C. Whether

Table 5. Content of nitrogen and non-protein nitrogen (mg/g H_2O) before and after ultracentrifugation.

	Before		After	
	N	NPN	N	NPN
Animal A	4.96	1.97	1.60	1.67
Animal B	5.45	1.64	1.58	1.56

Table 6. Average (3 animals) water, nitrogen, chloride, and sodium content of the supernatants of 3 and 70°C-treated homogenates before and after ultracentrifugation.

H_2O g/g			N mg/g H_2O			Cl mg/g H_2O			Na mg/g H_2O		
3°C	70°C	3°C	3°C	70°C	3°C	3°C	70°C	3°C	3°C	70°C	3°C
% NaCl			% NaCl			% NaCl			% NaCl		
3.7	3.7	0	3.7	3.7	0	3.7	3.7	0	3.7	3.7	0
Before ultracentrifugation											
0.932	0.949	0.972	5.52	1.96	3.93	22.3	22.0	0.136	14.7	14.7	0.159
After ultracentrifugation											
0.956	0.954	0.987	1.70	1.68	1.56	20.9	21.2	0.126	14.3	14.5	0.155

Table 7. Average (3 animals) potassium, calcium, magnesium, and zinc content of the supernatants of 3 and 70°C treated homogenates before and after ultracentrifugation.

K mg/g H_2O			Ca mg/g H_2O ($\times 10^3$)			Mg mg/g H_2O ($\times 10^3$)			Zn mg/g H_2O ($\times 10^3$)		
3°C	70°C	3°C	3°C	70°C	3°C	3°C	70°C	3°C	3°C	70°C	3°C
% NaCl			% NaCl			% NaCl			% NaCl		
3.7	3.7	0	3.7	3.7	0	3.7	3.7	0	3.7	3.7	0
Before ultracentrifugation											
1.52	1.53	1.48	9.23	10.02	5.57	78.2	81.0	61.3	5.74	0.648	4.52
After ultracentrifugation											
1.44	1.46	1.40	8.66	10.14	5.52	65.7	70.0	52.0	1.70	0.653	1.48

Table 8. Average (3 animals) percentage of the nitrogen and electrolyte content of supernatant fractions retained in solution after 24 hr of ultracentrifugation.

N			Cl			Na			K		
3°C	70°C	3°C	3°C	70°C	3°C	3°C	70°C	3°C	3°C	70°C	3°C
% NaCl			% NaCl			% NaCl			% NaCl		
3.7	3.7	0	3.7	3.7	0	3.7	3.7	0	3.7	3.7	0
31.0	85.7	39.6	93.6	96.3	91.9	96.7	98.1	97.4	94.7	95.4	94.8
Ca			Mg			Zn					
93.9	101.2	99.3	84.6	84.6	86.9	32.0	100.9	35.3			

this heat-stable protein represents a portion of the principal salt-soluble proteins or is a specific protein, awaits further study.

Table 8 shows the amount of nitrogen and electrolytes in ultracentrifugally obtained solutions expressed as the percentage of the amount originally present in the supernatant fraction of the homogenates. These figures represent the percentage of each electrolyte in the supernatant that was not bound. Nitrogen content ranged from 31.0 to 85.7%; chloride, from 91.9 to 96.3%; sodium, from 96.7 to 98.1%; potassium, from 94.7 to 95.4%; calcium, from 93.9 to 101.2%; magnesium, from 84.6 to 86.9%; and zinc, from 32.0 to 101.0%. The results show that of the electrolytes determined, only zinc was substantially and strongly associated, or bound, with water- and salt-soluble proteins.

DISCUSSION

The authors have undertaken herein to obtain new information on the role played by the addition of NaCl to meat in meat curing. Investigated were the binding of added sodium and chloride and the distribution of calcium, magnesium, and zinc in increasing concentrations of NaCl and at 3 and 70°C. Also, information was obtained on the amounts of electrolyte bound to water- and salt-soluble protein fractions.

The sodium and chloride results in Table

5 show that little or no binding of sodium or chloride occurred. Such binding should occur if published explanations of the effect of NaCl on the water retention and swelling of meat are valid, namely: a) that electrostatic binding of chloride ion causes increased repulsion of the peptide chains with subsequent absorption of water by capillary condensation (Hamm, 1957); b) that sodium and chloride absorption is an important factor (Sherman, 1962); and c) that sodium ions are preferentially absorbed by the protein imidazole groups (Mahon, 1961). Hamm's (1957) conclusion that preferential chloride ion absorption is important is based on the decreased pH that follows mixing NaCl with meat. Mahon's (1961) conclusion that preferential absorption of sodium ion occurs is based on shifts of titration curves in the acidic region and the additional sodium hydroxide that must be added to attain a given pH value in the presence of NaCl. Both authors measured hydrogen ion concentration potentiometrically and dealt with extremely small ionic shifts. A shift in pH of one unit, as from 5.0 to 6.0, a pH change larger than that achieved experimentally (Wierbicki *et al.*, 1957), results in a total change of 9×10^{-6} millimoles of hydrogen ion per ml, or the equivalent of 207×10^{-6} mg Na/ml and 319×10^{-6} mg Cl/ml, an amount too small to detect by direct analytical techniques. The present findings also do not support the conclusions of Sherman (1962) that sodium and chloride absorption occurs appreciably. Close examination of his results and method of measuring ion absorption raises two possible explanations of the variance of his conclusions with those indicated by the present evidence: 1) that he failed to take into account the water originally present in the meat in performing calculations and 2) that he assumed that ions present in the aqueous phase in the residue were bound to the meat proper, failing to calculate unbound electrolytes in this phase.

In any case, the present results show that the aforementioned explanations by Hamm (1957), Mahon (1961), and Sherman (1962) account only partly for the effects produced by NaCl. At 3°C, NaCl promoted the release of calcium, magnesium, and, to a lesser extent, zinc from the solid phase to the aqueous phase. The ionic movements found were of greater magnitude than the amounts of binding attributed to chloride and sodium ions. By comparing the amount of free ion released by the addition of 3.7% NaCl, as seen by reference to the analyses of ultracentrifuged extracts (Table 9), the amounts of calcium, magnesium, and zinc released were respectively 3.14×10^{-3}, 13.7×10^{-3}, and 0.22×10^{-3} mg/ml. These levels are fortyfold greater than amounts of bound chloride ion and sodium ion.

Heating at 70°C in the presence of no added NaCl promoted the release of all the magnesium and increased by 30% the amount of soluble calcium. A further increase, amounting to 5% of the bound calcium, was produced by adding 3.7% NaCl to the homogenate. The report of Hamm and Deatherage (1960) that free carboxyl groups are destroyed by heating may, in part, explain the release of magnesium and calcium on heating. Heating decreased the amount of zinc in the soluble phase. As shown by the data obtained by ultracentrifugation, some of the free zinc decreased on heating. Work of Edman (1959a,b) showing that zinc is bound to imidazole groups that are unaffected by heating (Hamm and Deatherage, 1960) may explain the continued retention of zinc on the proteins at elevated temperature. The zinc-containing enzymes (Berman, 1961) were presumably precipitated on heating, accounting for part of the decrease of soluble zinc. The report of Gurd and Wilcox (1956), stating that new binding sites may become available on heating, could explain the observed uptake of ionic zinc.

The present results open to question the mode of action attributed to the phosphates when used in conjunction with NaCl. Grau *et al.* (1953a,b) and Hamm (1956, 1957) credit the phosphates with sequestering calcium, magnesium, and zinc ions in raw meat. The results presented, at least in meat held beyond rigor, would tend to minimize the sequestering role of the phosphates, for at 3°C the addition of NaCl increases

by 28.6–33.6% the amount of soluble magnesium and calcium, with but 4 and 20%, respectively, remaining in the insoluble phase. With 47 and 95% of the zinc still in the insoluble phase at 3 and 70°C, respectively, the sequestering role of the phosphates may be limited to the removal of zinc. The effect of zinc in meat has been considered to be similar to that of calcium in that its binding to the structural proteins of meat has been assumed to have an adverse effect on water retention (Hamm, 1958, 1959). However, the direct, highly significant correlation found between water retention and zinc content, in contrast to the inverse relation found between water retention and either calcium or magnesium content (Swift and Berman, 1959), and, now, the contrast in binding between zinc and the other two ions observed in the present study, indicates that zinc differs in an important aspect from the two other ions. In previous studies (Hamm, 1955, 1958), zinc removal has been concomitant with calcium and magnesium removal, which could have obscured its role. The possibility exists that the enhanced hydration observed experimentally and attributed to calcium and magnesium removal may actually have consisted of a composite of effects in which the effect of calcium and magnesium removal could mask the effect of zinc removal, which could possibly have either a negative or positive effect on hydration. The specific effect of zinc on meat hydration remains in doubt.

The validity of treating the supernatant fractions as representative of the entire aqueous phases present in the residues, or throughout the homogenates, was supported by the results of the sodium and chloride analyses. At all concentrations of added NaCl, the analytical results for sodium and chloride were, within experimental error, comparable to the calculated values, i.e., in each case, the water content of the supernatant fraction and the water content of the residue, multiplied by the concentration of NaCl, equaled the amount of NaCl added. Nevertheless, the analyses do not permit the validity of the assumption to be rigorously proven, since the possibility exists that part of the water phase in residues may not have been in equilibrium with the supernatant fractions, in which case the sodium and chloride this water would have contained at equilibrium could actually be bound. It is also recognized that error in determinations of free electrolytes, using the ultracentrifugation method, could result from denaturation of protein or dissociation of electrolyte due to protein sedimentation.

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A RESEARCH NOTE

Enzyme-Inactivation Studies on Irradiation-Sterilized Cod Fillets[a]

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Storage of irradiated seafoods above freezing temperature requires a pre-irradiation heat treatment to prevent autolysis. The importance of inactivation of naturally occurring autolytic enzymes in irradiated meat to be stored above the freezing point has been clearly demonstrated (Pratt and Ecklund, 1956; Drake *et al.*, 1957; Cain *et al.*, 1958; Chiambalero *et al.*, 1959; Pearson *et al.*, 1960; Artar *et al.*, 1961). No report was available on the inactivation temperature of seafood enzymes. Since our work was concerned primarily with the development of an irradiated cod cake, time-temperature heat treatments for inactivation of cod muscle enzymes were determined.

Fresh Pacific cod (*Gadus macrocephalus*) were landed and filleted at Astoria, Oregon. The fillets were ground in a stainless-steel meat grinder to provide a homogeneous sample. The ground cod was put in a hand-operated upright sausage stuffer (F. Dick Co.), stuffed into sausage casing (2½ × 32 E P Fibrous Visking Co.), quick frozen at −18°F, and held at 0°F, according to the procedure reported by Artar *et al.* (1961). Just prior to heat inactivation, the casings were peeled off and the ground sample sliced with a band saw into cakes ¾-inch thick. The cakes were placed in mylar-polyethylene bags and heated in a hot-water bath to internal temperatures of 150, 160, and 170°F (come-up times of 15, 18, and 21 min, respectively) and held for specified time intervals as shown in Table 1. The water-bath temperature was maintained at 10°F above the desired internal temperature, which was measured with thermocouples inserted in the fish cakes. At the end of each time-temperature period, the bags were placed in a cold-water bath (40°F) until the internal temperature was 100°F; approximately 7 min was required. The bags were heat sealed, placed in 307 × 202 cans, hermetically sealed, and quick frozen at −18°F. They were shipped under dry ice, via Railway Express, to the Materials Testing Reactor at Arco, Idaho, for irradiation at 4.5 megarad and returned to our laboratory under the same conditions. Irradiation was effected in water maintained at 78°F, and the average dose rate was 4.76 × 10^6 rad/hr. The samples were stored at 0 and 100°F for 0, 14, and 30 days. The unirradiated control samples were held at 0°F. The irradiated and control samples were analyzed for increase in amino nitrogen by the copper method of Pope and Stevens (1939).

Before chemical analysis, the fish cakes were reground and mixed with the liquid in the bags. Forty grams of fish and 80 ml of distilled water were blended in a Virtis blender for 3 min, centrifuged for 5 min at 1800 rpm, and filtered if necessary. A 15-ml sample was used to determine amino nitrogen. Duplicate samples were analyzed, and the average was reported as mg amino nitrogen per 100 g of fish.

Results are shown in Table 1. Fig. 1 shows the "break point"—the point at

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Table 1. Amino nitrogen content [a] of cod as affected by preheating, irradiation, and storage.

Irradiation level (mrad)	Storage temp. and time	Pre-irradiation heat treatment (°F; sec)										
		150° 15	150° 60	150° 180	150° 300	160° 15	160° 60	160° 180	160° 300	170° 15	170° 60	170° 180
0	0°F, 0 time	32.9 [a]	30.8	30.8	28.7	28.7	27.7	28.4	29.4	30.0	31.6	28.8
4.5	0°F, 0 time	31.5	32.0	34.0	32.7	31.9	34.3	31.2	30.8	33.1	33.3	29.4
4.5	100°F, 2 weeks	67.4	41.0	42.7	36.1	36.1	36.4	35.0	35.7	35.7	35.3	39.8
4.5	100°F, 4 weeks	126.0	62.1	56.8	38.6	41.0	40.3	37.4	37.5	38.2	40.6	38.2

[a] Mg amino nitrogen/100 g fish.

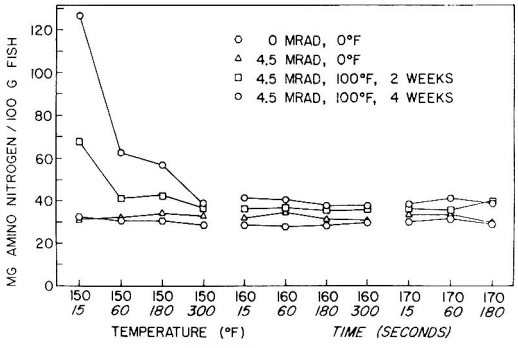

Fig. 1. Amino nitrogen content of cod as affected by preheating, irradiation, and storage.

which enzyme inactivation is believed to occur.

Release of amino acids is dependent upon pre-irradiation heat treatment, storage time, and storage temperature. According to the conditions of this experiment, it appears that a minimum time-temperature treatment of 300 sec at 150°F or 15 sec at 160°F is required prior to irradiation for inactivation of enzymes in cod muscle.

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Rehydration of Freeze-Dried Pork as Related to pH and Protein Denaturation[a]

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SUMMARY

In order to determine the influence of pH on percentage rehydration and the degree of protein denaturation caused by freeze dehydration, 70 freeze-dried fillets taken from the longissimus dorsi muscle of three hogs were utilized. Percentage rehydration of the freeze-dried fillets ranged from 48.5 to 92.4%, with a mean of 73.8%, and was not influenced significantly by either pH of the rehydrating solution or pH of the rehydrated meat under the conditions of this trial. Fat content had little effect on percentage rehydration. An increase in pH was noted during the dehydration process, but trapping of the acidic volatiles and adding them back during rehydration restored the original pH. The qualitative amino acid composition of the rehydration solution was not influenced by pH. However, a change in ionic strength varied the qualitative amino acid composition. The sarcoplasmic protein fraction noticeably decreased in nitrogen content on freeze-dehydration and rehydration, indicating that some denaturation was occurring.

INTRODUCTION

Dehydration by freeze-drying produces dehydrated foods of the highest quality. Despite all of the advantages of freeze-drying, Auerbach *et al.* (1954) found that freeze-dried beef rehydrated to only 80–90% of the original water content. Brooks (1958) reported that reconstituted freeze-dried meat was tougher and had a drier texture than control meat. According to Deatherage and Hamm (1960), freeze-drying alters the water-holding capacity of meat.

This study was made to investigate the effect of pH on the percentage rehydration of freeze-dried pork and to determine the degree of protein denaturation and its relation to rehydration.

EXPERIMENTAL

Experimental meat. The longissimus dorsi muscle was obtained from hogs slaughtered in the Michigan State University abattoir. No attempt was made to relate treatment effects to the previous history of the animal, since meat from the same animal served as the untreated control in all studies.

Sample preparation. All separable fat and visible connective tissue were removed from the excised muscle samples. One-hundred-gram samples were removed from the posterior, middle, and anterior portions of the muscle. The composite sample was ground twice through a 1-cm plate and twice through a 2-mm plate of a Hobart grinder. The grinder head and plates were prechilled to 4°C in all cases to prevent heat denaturation during grinding.

The remainder of the muscle was sliced into 26 fillets, ¼-inch thick, weighed to the nearest tenth of a gram, wrapped in aluminum foil, and frozen in an air-blast freezer held at approximately −29°C for 3 hr. The frozen samples were freeze-dried for 20–24 hr in a Stokes freeze-dryer, Laboratory Model 2003 F-2, using a vacuum of 150 μ Hg, with a plate temperature ranging from 28 to 30°C. Upon removal from the Stokes apparatus, the samples were immediately reweighed, wrapped individually in aluminum foil, and stored under nitrogen in a desiccator at room temperature. Length of storage never exceeded four weeks.

Rehydration of fillets. Fillets of known weight were immersed with glass weights in 150 ml of buffer solution in covered casserole dishes. The fillets were rehydrated in duplicate, 4½ hours at 4°C, in various buffers ranging in pH from 2.55 to 9.05.

The rehydrated pork was blotted with Whatman No. 1 filter paper for ½ min on each side to remove excess moisture. Samples were weighed, and the percentage rehydration was calculated ac-

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cording to the formula: $W_R/W_L \times 100$, where W_R = g moisture regained in rehydration, and W_L = g moisture lost in freeze-dehydration.

The term buffer solution as used throughout this discussion refers to the solution utilized for rehydrating the fillets, whereas the term rehydration solution refers to the buffer solution at the end of rehydration. Obviously, the buffer solution contains no soluble material from the meat, whereas the rehydration solution contains material leached from the meat during rehydration.

Chemical analyses. Fat and moisture were determined by the methods of Benne *et al.* (1956). Protein nitrogen analyses were performed, in duplicate, by the micro-Kjeldahl method of the American Instrument Company (1961). Non-protein nitrogen was determined according to the procedure outlined by Hegarty *et al.* (1963). Nitrogen contents were reported as mg of protein nitrogen or non-protein nitrogen per ml of solution or per g of solids. All pH measurements were made with a Beckman pH meter, Model G. The electrodes were placed directly into the ground-meat sample or protein solution.

Protein fractionation. The protein fractionation procedure was adapted from that of Hegarty *et al.* (1963), with the principal change occurring in the comminution procedure. The samples were blended in a Waring blender for a 10-second burst followed by a 3-minute rest period, repeated 3 times. In the present study, a phosphate buffer of pH 7.6, $\mu = 0.05$ (0.156*M* K_2HPO_4; 0.0035*M* KH_2PO_4), was used for the quantitative determination of sarcoplasmic protein nitrogen, and a KCL-bicarbonate buffer, pH 8.25, $\mu = 0.53$, (0.5*M* KCL; 0.03*M* $NaHCO_3$) was used for the quantitative determination of fibrillar protein nitrogen.

Amino acid composition of rehydration solution. The total amino acid content, the free amino acid content, and the non-protein nitrogen amino acids of the rehydration solution were separated on a one-dimensional descending chromatograph according to the procedure of Moffat and Lytle (1959). The amino acid fractions were obtained by acid hydrolysis.

Volatile loss detection. A 20-g portion of the ground fresh meat was blended with 80 ml of deionized water for 1 min in a Waring blender adjusted with a Powerstat transformer setting of 60. The pH of the slurry was taken. The slurry was transferred to a 1000-ml round-bottom flask, which was then slowly rotated in an ethanol-dry-ice bath, causing the meat slurry to be frozen as a thin shell on the surface of the flask. The flask was attached to a vacuum distillation apparatus consisting of one ethanol-dry-ice trap and two liquid-nitrogen traps. A vacuum ranging from 170 to 50 μ Hg was created, and complete dehydration occurred within 6–9 hr.

The ethanol-dry-ice trap contained all of the water removed from the meat slurry. The water was thawed, and its pH was recorded. The volatiles trapped in the two liquid-nitrogen traps were distilled into the thawed water, using nitrogen to flush out the containers. After each trap had been distilled, the pH of the solution was taken. The solution, consisting of all volatile constituents removed during dehydration of the slurry, was added to the dehydrated meat solids, and the pH was measured again.

RESULTS AND DISCUSSION

Duplicate fillets were rehydrated in either 10 or 11 different buffers of 0.05 μ covering a pH range of 3.62–9.05. Four samples were rehydrated in a buffer of 0.1 μ, pH 3.05, and 2 samples were rehydrated in a buffer of 0.1 μ, pH 2.55. The means and standard deviations for pH and percentage rehydration are presented in Table 1. The analysis of variance between percentage rehydration for different loins indicated a significant difference between the means for percentage rehydration of the three loins used in this study. The significant deviation between loins may have been caused by breed differences or possibly by an individual reaction to the dehydration process. Since the pork used fell within a rather narrow initial pH range, the effects of wide pH differences in the tissues were not ruled out.

Table 2 summarizes the correlation coefficients of pH versus percentage rehydration. The association between percentage rehydration and either pH of the buffer solution or pH of the rehydrated meat was not statistically significant. Thus, freeze-dried pork showed no optimum pH for rehydration. This is in contrast to findings of Auerbach *et al.* (1954), who reported that the rehydration of freeze-dried beef was most complete at pH 7.00. Thus, a difference is indicated between freeze-dried pork and beef, which may possibly be attributed to different physiological characteristics of the two species. A direct correlation between pH of the buffer solution and the pH attained by the rehydrated pork was observed for all three loins (pooled data, $r = 0.82$). This relationship was not sur-

Table 1. Data showing pH of rehydrating buffer, pH of rehydrated chops, and loins and for pooled information.

	Loin 1			
	pH buffer solution[a]	pH rehydrated meat	Percentage rehydration	pH buffer solution[a]
	3.05	4.65	76.78	3.05
		4.68	83.10	
	3.73	4.85	76.30	3.73
		4.98	53.59	
	4.35	5.09	61.25	4.35
		5.03	70.34	
	5.85	....	....	5.85
		....	....	
	6.10	5.65	61.17	6.10
		5.62	69.78	
	6.70	5.82	66.08	6.70
		5.80	80.77	
	7.19	5.90	66.40	7.19
		6.00	81.41	
	7.30	5.48	62.95	7.30
		5.50	61.36	
	7.65	5.72	70.09	7.65
		5.60	71.43	
	7.72	5.61	58.99	7.72
		5.65	59.05	
	9.18	6.68	68.41	9.18
		6.55	73.85	
	Deionized H_2O	5.69	48.54	Deionized H_2O
	pH 7.00	5.65	61.33	pH 7.00
Means	6.32	5.55	67.41*	6.32
Standard deviations	1.81	0.51	8.86	1.74
Pooled data—Mean	....	....	....	....
Standard deviation	....	....	....	....

[a] pH buffer solution was the same for duplicate sample.
* Significantly different at 5% level.
** Significantly different at 1% level from underscored means.

percentage of rehydration with means and standard deviations by

Loin 2			Loin 3	
pH rehydrated meat	Percentage rehydration	pH buffer solution[a]	pH rehydrated meat	Percentage rehydration
4.55	66.47	2.55	4.79	83.28
4.20	86.06		4.90	58.46
4.99	84.24	3.62	4.90	66.47
4.95	81.08		4.99	70.98
5.40	82.47	4.28	4.62	80.12
5.30	83.52		4.60	74.47
5.75	92.41	5.85	5.15	71.88
5.75	91.54		5.10	75.00
5.86	84.70	6.68	5.20	65.71
5.86	79.08		5.15	77.61
5.80	73.96	6.95	5.82	69.19
5.83	70.35		5.85	64.55
5.60	81.23	7.15	5.32	74.47
5.63	75.43		5.29	70.05
5.30	84.19	7.58	5.95	67.76
5.32	85.80		5.92	82.94
5.83	90.07	7.61	5.60	77.47
5.80	62.07		5.71	65.56
5.35	72.50	7.85	5.35	80.00
5.39	82.07		5.48	68.84
6.20	75.00	9.05	6.19	74.74
5.80	85.07		6.35	70.04
5.25	82.58	Deionized H_2O	5.75	78.95
5.45	82.70	pH 7.00	5.75	76.51
5.47	80.61**	6.35	5.41	72.71*
0.44	7.38	1.85	0.48	6.18
....	....	6.38	5.47	73.75
....	....	1.80	0.48	9.25

Table 2. Correlation coefficients between pH of buffer solution, pH of rehydrated meat and percentage rehydration.

Loin	Correlation coefficients: pH buffer solution vs. percentage rehydration	Correlation coefficients: pH rehydrated pork vs. percentage rehydration
1	−.22	−.06
2	−.08	−.03
3	0.09	−.07
Pooled data	−.07	−.08

prising, for the pH of meat would be expected to change with the addition of an acidic or basic solution.

The experimental data indicated that freeze-dried pork rehydrated to a much lower level than beef. Percentage rehydration of freeze-dried pork ranged from 48.5 to 92.4%. The mean percentage rehydration of 70 samples was 73.8% (±9.3%). The distribution of percentage rehydration is summarized in the histogram in Fig. 1.

According to Tappel *et al.* (1955), 1-inch pieces of the biceps femoris of beef attained an 80–90% level of rehydration. Harper and Tappel (1957) stated that freshly prepared freeze-dried beef rehydrated to a maximum level of 80–100% of its original water content. Although freeze-dried pork is very similar to freeze-dried beef in structure and texture, there appears to be a noticeable difference in their rehydration characteristics.

Results in Table 3 show that loin 1, which had a low fat content, did not rehydrate to a higher level than loin 3, which had a high fat content. According to Orme *et al.* (1958), the fat content of beef longissimus dorsi ranged from 1.90 to 11.21%. The mean percentage fat averaged 4.25 and 8.59% for good and prime steers, respectively. Pearson *et al.* (1962) found that the percentage fat of pork longissimus dorsi ranged from 2.14 and 8.14%. Since the percentage intramuscular fat in beef and pork longissimus dorsi does not differ greatly, no marked difference in rehydration would be expected between beef and pork. It is also interesting to note that loin 2, which had an intermediate fat content, rehydrated more completely than either of the others.

Since the fat content of the individual rehydrated fillets of loins 1, 2, and 3 had been calculated from the fat content of the fresh control, a fourth loin was freeze-dried and rehydrated. Results showed that the correlation ($r = 0.17$) between fat content and percentage rehydration was not statistically significant. Fat content, therefore, does not appear to influence percentage rehydration greatly.

The influence of freeze-drying on the pH of pork rehydrated in deionized water is shown is Table 4. Seven of eight rehydrated fillets achieved a higher pH than that of the controls.

To determine whether the change in pH was caused by protein denaturation or a loss of volatile constituents, the volatiles were collected and added back during rehydration. Results in Table 5 indicate that the volatile losses are responsible for most

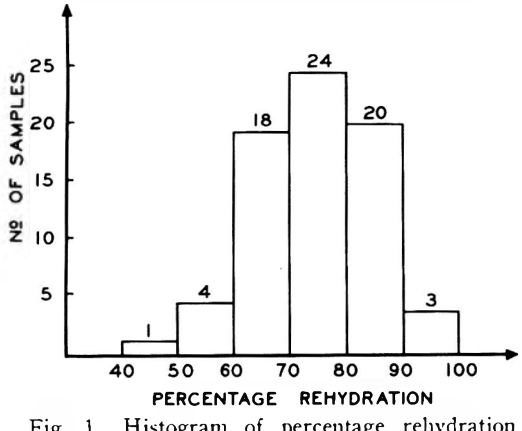

Fig. 1. Histogram of percentage rehydration for freeze-dried pork fillets.

Table 3. Mean percentage rehydration and percent fat content of fresh longissimus dorsi.

Loin	% fat	Mean percentage rehydration
1	2.40	67.41±8.86
2	2.94	80.61±7.38
3	4.26	72.71±6.18

Table 4. Influence of freeze-drying on the pH of rehydrated pork.

Loin	pH: Fresh	pH: Rehydrated	pH: Duplicate[a]
1	5.32	5.69	5.65
2	5.35	5.25	5.45
3	5.51	5.75	5.75
4	5.29	5.45	5.45

[a] Samples rehydrated in deionized water at 4°C.

of the changes in pH, and that such losses, if trapped and used in reconstitution of the dried meat solids, would restore the original pH of the fresh-meat slurry. The differences observed between trials (Table 5) may be explained by the varying experimental conditions. The rise in pH that occurred when pork was rehydrated indicated that acidic volatiles are removed during the freeze-drying process. The nature of the volatiles was not studied in this investigation.

Table 6 presents the means and standard deviations for the nitrogen content of the various protein fractions extracted from the freeze-dried loins. The large standard deviation obtained for the soluble fibrillar protein content made it impossible to arrive at any direct conclusions on the basis of these results. The wide range in the data could have resulted from an incomplete extraction of the fibrillar proteins. In the present study the freeze-dried rehydrated fillets were hand-minced, so the size of the mince varied. In further studies, the experimental error could probably be reduced by increasing the size of the fillets so as to permit mechanical grinding.

Table 7 compares the nitrogen contents of the protein fractions extracted from fresh and freeze-dried loins. If one assumes

Table 5. Influence of freeze-drying on the loss of volatiles from pork.

Conditions	Trial (1)	Trial (2)	pH difference [a]
Pressure	170–70 μ Hg	50 μ Hg	
Dehydration time	9 hr	6 hr	
pH			
Meat slurry, fresh	5.23	5.15	
Volatile fractions			
Trap 1: Solution 1 (thawed water)	6.45	5.62	0.83
Trap 2: Solution 2 (Solution 1 + volatiles of 1st liquid N_2 trap)	5.68	5.30	0.38
Trap 3: Solution 3 (Solution 2 + volatiles of 2nd liquid N_2 trap)	4.49	5.62	−1.13
Meat slurry, reconstituted (dehydrated solids + solution 3)	5.28	5.18	

[a] Difference in pH between trial 1 and trial 2 (trial 1 − trial 2).

Table 6. Means and standard deviations for the nitrogen content of the protein fractions extracted from freeze-dried loins expressed as mg N/g solids.

Item	Means and standard deviations Loin 1	Loin 2	Loin 3	Pooled data
Sarcoplasmic protein nitrogen	26.93±4.01	27.23 ±4.79	28.04±7.24	27.41±5.58
0.53 μ soluble protein nitrogen	35.07±6.15	33.48 ±5.03	35.99±7.89	34.84±6.56
Soluble fibrillar protein nitrogen	8.29±6.21	6.57 ±4.62	8.08±4.97	7.63±5.34
Non-protein nitrogen	12.88±1.82	10.72**±1.69	13.91±3.05	12.63±2.53
Rehydrating solution protein nitrogen	2.25± .654	2.95 ± .981	2.63± .615	2.62± .821
Rehydrating solution non-protein nitrogen	4.49± .556	5.34 ± .686	5.62± .740	5.17± .820

** Significantly different at 1% level from the underlined observations. All other values were not significantly different.

Table 7. A comparison of the nitrogen content (mg N/g solids) of the protein fractions extracted from fresh and freeze-dried loins.[a]

Protein fractions	Loin 1		Loin 2		Loin 3	
	Fresh	Freeze-dried	Fresh	Freeze-dried	Fresh	Freeze-dried
Sarcoplasmic protein nitrogen	30.45	26.93	47.51	27.23	35.04	28.04
0.53 μ extractable protein nitrogen	36.98	35.07	57.74	33.48	39.11	35.99
Soluble fibrillar protein nitrogen	6.53	8.29	10.23	6.57	4.07	8.08
Non-protein nitrogen	14.31	12.88	13.95	10.72	16.47	13.91

[a] Mean value of nitrogen content for freeze-dried loins is recorded.

the nitrogen content of the rehydration solution (Table 6) to be composed entirely of sarcoplasmic protein nitrogen, a decrease in the sarcoplasmic protein content on freeze-dehydration is evident. When the non-protein nitrogen content of the rehydration solution is considered, the total non-protein nitrogen was greater than that of the controls.

Denaturation of the sarcoplasmic proteins of pork appeared to result from freeze-drying as evidenced by a decrease in the concentration of nitrogen in the sarcoplasmic protein fraction. The freeze-dehydration process may in some way affect the bonds that are due to electrostatic interaction between polar groups and to the van der Waals forces between non-polar groups of the meat proteins.

The influence of pH on the amino acid composition of the rehydration solutions was investigated in loin 4. The amino acids of the rehydration solutions (buffers at pH 2.55, 5.85, 9.05 and deionized water) were fractionated. Results indicated that the rehydration solutions of deionized water, pH 5.85 and pH 9.05, were similar in their qualitative amino acid composition. Thus, pH did not appear to influence the qualitative amino acid composition of rehydration solutions. The ionic strength of the pH 2.55 buffer was 0.1, whereas all other rehydration solutions investigated had an ionic strength of 0.05. A change in ionic strength greatly influenced the fingerprinting of the amino acids.

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Changes of Characteristics of Starch during Gelatinization in the Presence or Absence of Fatty Acid

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SUMMARY

To study the effects of lipid on the gelatinization of starch, changes in its characteristics were investigated by gelatinizing the potato starch in amylograph in the presence or absence of linoleic acid. Added linoleic acid resulted in an increase in the temperature at which both viscosity and size of starch granules increased, but did not affect the temperature at which X-ray pattern and digestibility changed from those of raw starch to those of gelatinized starch.

INTRODUCTION

Since lipid or monoglyceride has come to be used for the manufacture of mashed potato and in the retardation of bread staling, attention has been attracted to the relation between lipid and starch gelatinization. For example, Schoch (1945), Tamura *et al.* (1957), and Fujii and Oba (1962) reported that extraction of lipid made starch fragile, changing its rheological properties, such as the gel strength, amylogram, and viscosity of gelatinized starch paste.

Osman *et al.* (1960, 1961), Fujii and Oba (1962), and Gray and Schoch (1962) investigated the effects of glycerides, surfactants, and fatty acids on the gelatinization of starch, and found that a small amount of lipid or a surfactant added to starch paste inhibits the swelling. In their investigations, the reason was reported to be that the linear-chain molecules form a helical configuration with the linear-chain molecules of starch and restrict the passage of water that is necessary for the swelling of starch granules.

On the other hand, there are many investigations about the mechanism of starch gelatinization. For instance, Hizukuri *et al.* (1960) reported in detail the relation between the viscosity change and micelle structure of starch during its gelatinization. But these reports were made without addition of any lipid material.

The present study was made to investigate the relation among the characteristics of starch (viscosity, size, digestibility, X-ray diffraction pattern) by gelatinizing the starch in the presence or absence of lipid.

EXPERIMENTAL

Material. Potato starch (Japanese Pharmacopoeia) was used in this study.

Amylograph. The standard-model amylograph (Brabender O.H., Duisburg-on-Rhine) was used. Twenty grams of potato starch was slurried in 370 ml of distilled water at 30°C and transferred to the cup of the amylograph. The slurry was heated to 92°C, maintained there for 10 min, and then cooled.

The amylograph in the presence of lipid was performed as follows. Linoleic acid was dissolved in 10 ml methanol and added to 150 ml of a starch suspension containing 20 g of starch. This suspension was stirred thoroughly, and stirring continued for 1 hr at 30°C, followed by pouring it into the amylograph cup with sufficient distilled water to give a final volume of 370 ml.

X-ray study. Potato starch for X-ray study was prepared according to the method of Hizukuri *et al.* (1960). About 10 ml of starch paste was taken and poured immediately to 200 ml methanol. The resulting precipitate was washed twice with ether, dried under reduced pressure, powdered, and stored in a desiccator.

The X-ray apparatus was Isodebyflex II-type (Seifert Co.). Operating conditions were as follows: X-ray, Cu Kα ray (wavelength 1.5418 Å) excited at 35,000 V; tube current, 15 ma; Cu Kβ ray was eliminated by a Ni-filter; width of receiving slit, 0.5 mm; time constant, 1 second; sensitivity, 1,000 counts per second; scanning speed of goniometer, 1° per minute; chart speed, 10 mm per minute. The peaks in X-ray diffraction patterns were designated according to the definitions of Hizukuri and Nikuni (1957).

Digestibility. The digestibilities of starch taken from amylograph were measured by the method of Kihara and Kawase (1949). Takadiastase was added to starch paste and incubated 3 days at 37°C. The resulting reducing sugar and total starch were determined with 3,5-dinitrosalicylic acid and anthrone method, respectively.

$$\text{Digestibility (\%)} = \frac{\text{resulting reducing sugar (mg glucose)}}{\text{starch (mg glucose)}} \times 100$$

Appearance and size of starch granules. The changes of appearance and polarization crosses of starch granules during its gelatinization were observed under the microscope. Diameters of starch granules were calculated by measuring 50 granules by photomicrograph.

RESULTS

Amylogram of potato starch in the presence or absence of linoleic acid. Fig. 1 is an amylogram of potato starch as well as its changes by addition of linoleic acid.

Changes similar to those reported previously (Osman and Dix, 1960; Gray and Schoch, 1962) were obtained by the addition of fatty acid, i.e., rise in temperature of viscosity increase.

X-ray diffraction patterns. Figs 2 and 3 show the changes of crystal structure during gelatinization. These figures must be considered along with the change of viscosity shown in Fig. 1, namely, that in the absence of fatty acid both viscosity and crystal structure changed at 65°C, whereas in the presence of linoleic acid the viscosity did not increase at 65°C though the crystal structure changed. That is, the addition of linoleic acid changes the temperature of viscosity increase but not the temperature at which the change in crystal structure of potato starch occurred.

Digestibility. Samples prepared for X-ray analysis were also used to measure digestibility, giving the result in Fig. 4. The figure clearly shows that digestibility increased before a viscosity change was observed, and that the presence of linoleic acid retarded the temperature of viscosity increase but not the temperature of digestibility increase.

Size. A change in size during gelatinization was observed under the microscope. The photomicrographs are shown in Figs. 5–7.

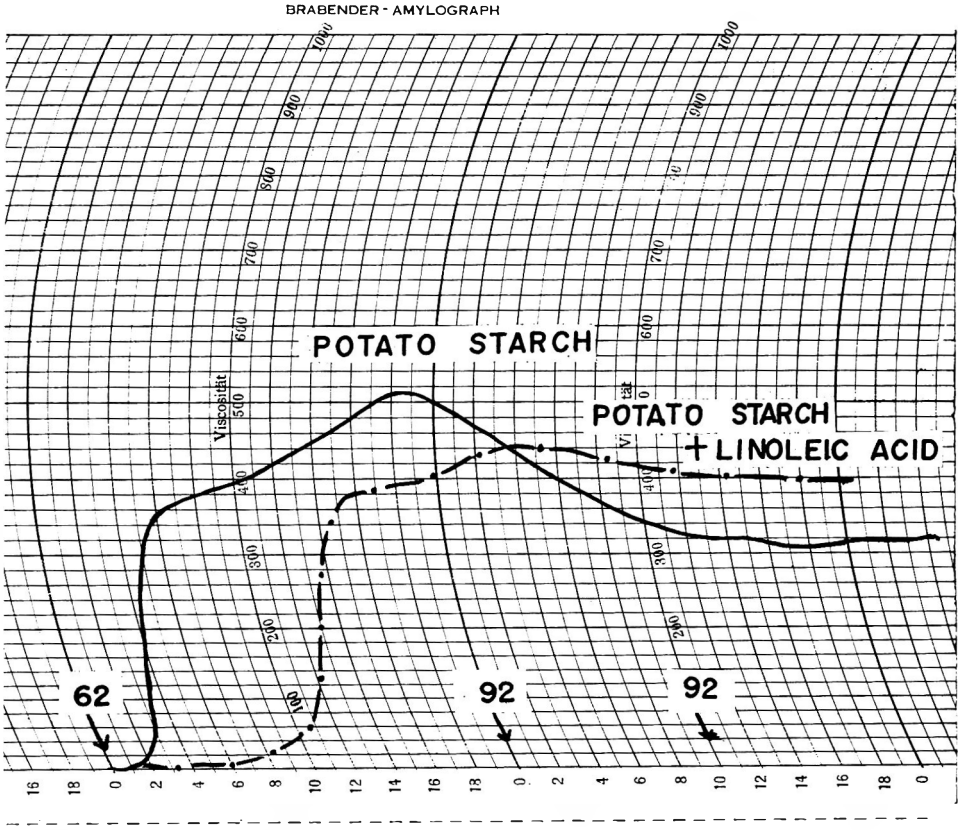

Fig. 1. Amylogram of potato starch in presence and absence of linoleic acid. 120 mg of linoleic acid was added to 20 g of potato starch suspended in 370 ml of water.

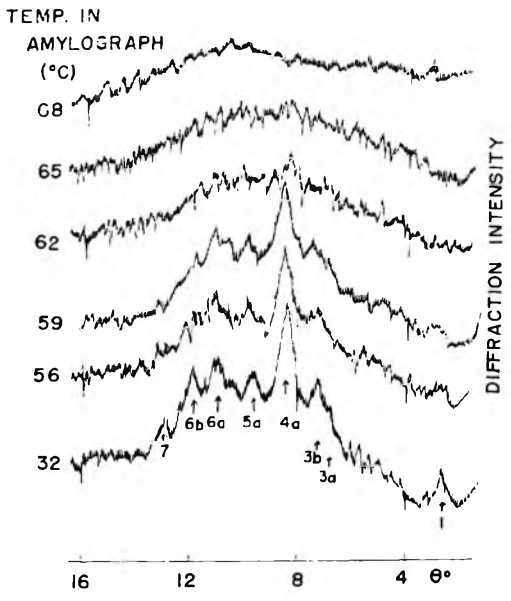

Fig. 2. Change in X-ray patterns of potato starch during gelatinization.

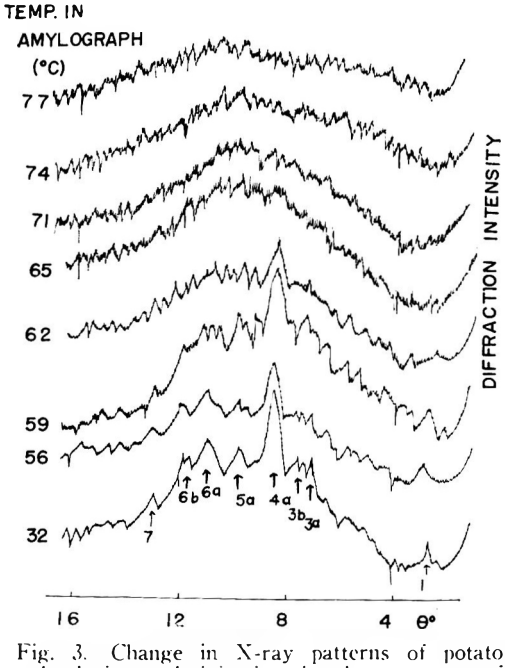

Fig. 3. Change in X-ray patterns of potato starch during gelatinization in the presence of linoleic acid.

When temperature rose above 62°C, the effect of linoleic acid was observed in the appearance of starch granules. That is, when the granules were heated in the absence of linoleic acid, they started to swell at 62°C, which is the temperature of initial increase in viscosity, and soon lost the structure and disrupted at 68°C. On the other hand, the granules, gelatinized in the presence of linoleic acid, gradually increased in size but did not lose their form even at such a high temperature as 86°C.

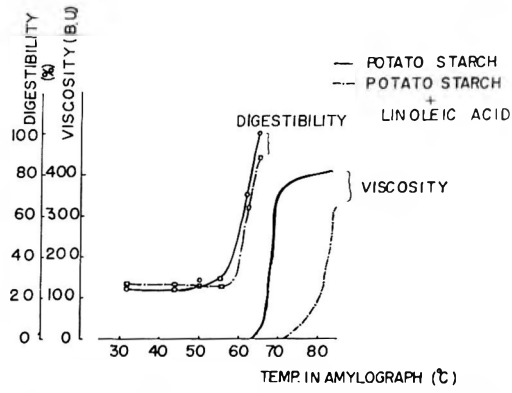

Fig. 4. Increase in viscosity and digestibility with temperature.

The change in size was also measured during gelatinization, giving the results in Fig. 8.

From Fig. 8 it is clear that, in the presence of linoleic acid, starch granules swell less than in water alone at a temperature above 62°C.

Birefringence. No difference in birefringence of starch was observed in granules gelatinized in the presence or absence of linoleic acid (Fig. 9).

However, Fig. 9 shows that the polarization crosses vanished as the starch granules swelled, independent of temperature or the presence of linoleic acid.

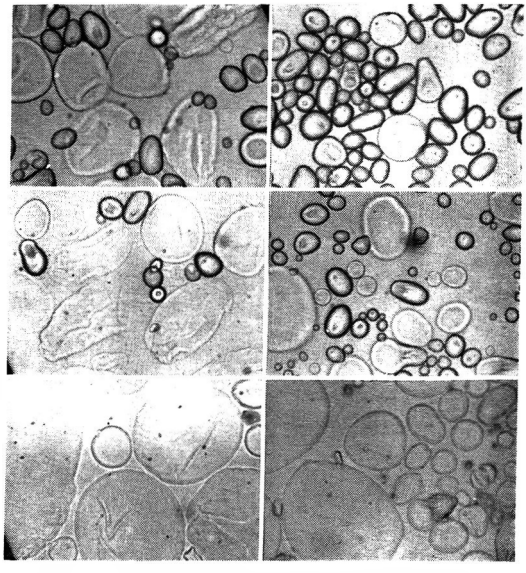

Fig. 5. Changes in appearance of starch granules during gelatinization. Left, potato starch; right, potato starch plus linoleic acid. Top, 62°C; middle, 65°C; bottom, 68°C.

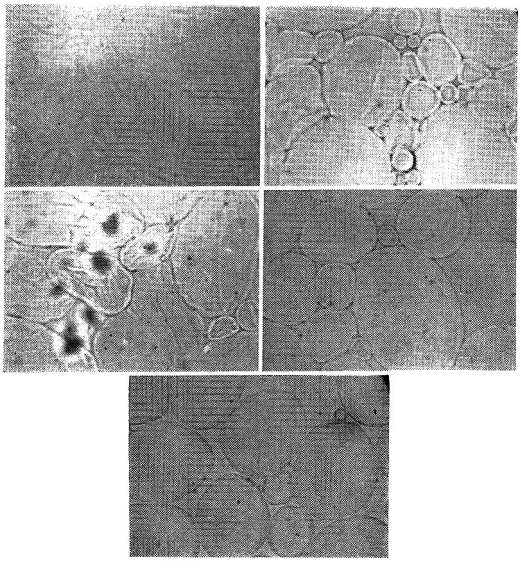

Fig. 6. Changes in appearance of starch granules during gelatinization. Left, potato starch; right, potato starch plus linoleic acid. Top, 71°C; middle, 74°C; bottom, 77°C.

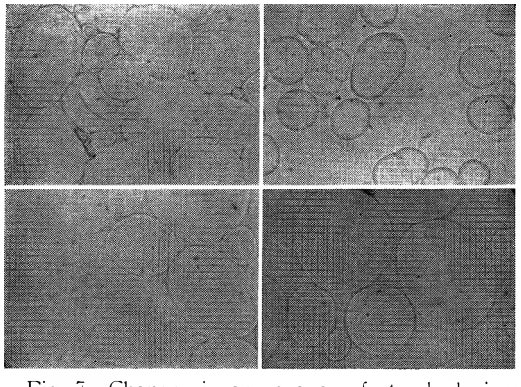

Fig. 7. Changes in appearance of starch during gelatinization of potato starch in the presence of linoleic acid. Top: left, 80°C; right, 83°C. Bottom: left, 86°C; right, 89°C.

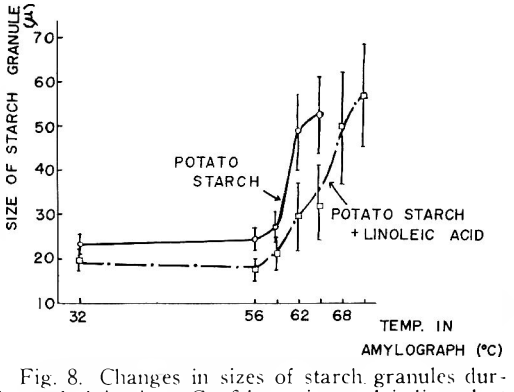

Fig. 8. Changes in sizes of starch granules during gelatinization. Confidence interval indicated at 5% level of significance.

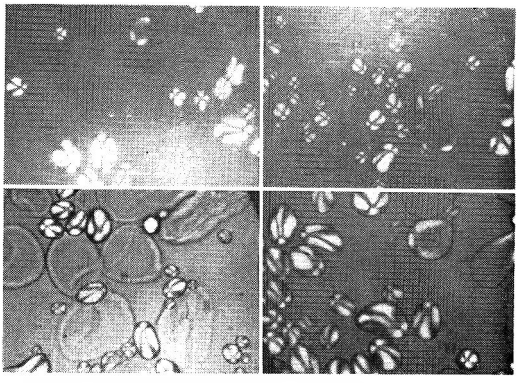

Fig. 9. Birefringence of starch granule. Left, potato starch; right, potato starch plus linoleic acid. Top, 56°C; bottom, 62°C.

DISCUSSION

Hizukuri *et al.* (1960) reported in detail the relation between the change of viscosity and that of X-ray diffraction pattern, using the VI-viscograph. But the present study employed the amylograph (Brabender) instead of viscograph, which caused a little difference in the results. But most of the results coincided with those of Hizukuri's researches, as follows: the firm crystal structure of raw starch becomes loose with increasing temperature, and the typical X-ray pattern of gelatinized starch is observed at a temperature of maximum viscosity.

However, of most interest in the present study is the new finding that, when starch is gelatinized in the presence of fatty acid, the change of X-ray pattern (i.e. change in micelle structure) does not relate to that of viscosity in amylogram.

The results concerning this relation are summarized in Table 1, which clearly indicates that X-ray pattern turned from B-type (raw potato starch) to V-type (gelatinized starch) at 65°C irrespective of the presence or absence of fatty acid. However, viscosity in the amylograph increases at 65°C in the absence of fatty acid but at 74°C in the presence of it.

As shown in Figs. 5–7 (photomicrograph), starch granules start to swell at 62°C, and if fatty acid is absent they swell rapidly thereafter, with resultant rapid disruption of swelled granules, but if fatty acid is present they swell gradually without being disrupted at all. In agreement with studies

Table 1. Change of potato starch during gelatinization.

Temp. (°C)	In absence of linoleic acid				In presence of linoleic acid			
	Viscosity (B.U.)	X-ray pattern	Digestibility (%)	Size (μ)	Viscosity (B.U.)	X-ray pattern	Digestibility (%)	Size (μ)
32	0	B	24.5	25.4	0	B	27.0	22.0
56	0	B	28.1	24.2	0	B	27.2	16.3
59	0	B	....	27.5	0	B	....	22.1
62	0	B	70.7	49.2	0	B	63.4	30.1
65	10	V	....	53.0	0	V	....	32.9
68	140	V	105.4	....	0	V	88.7	50.8
71	350	V	....	....	0	V	....	57.5
74	370	V	....	....	20	V	....	....
77	395	V	....	....	50	V	....	....

of Strandine *et al.* (1951) and Gray and Schoch (1962), this firmness of starch granules gelatinized in the presence of fatty acids seems also to support the discussion that fatty acid is supposed to be involved in helical configuration with linear molecule of starch and to form a persistent outer layer to restrict the passage of water, hence slowing the swelling of granules.

On the other hand, the disappearance of birefringence is usually used as a measure for gelatinization. The birefringence is reported to be due not to the configuration of micelle itself but to the orientation of the micelle (Hizukuri *et al.*, 1959). As seen in Fig. 9, the degree of swelling of starch granules is the most influential factor in the disappearance of birefringence, irrespective of the presence or absence of lipid during gelatinization.

As to digestibility, Fig. 4 shows that added fatty acid did not change the temperature at which the initial increase of digestibility was observed.

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Prevention of Pale, Soft, Exudative Porcine Muscle Through Partial Freezing with Liquid Nitrogen Post-Mortem[a]

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SUMMARY

Liquid nitrogen ($L\text{-}N_2$) treatment over a wide range of immersion rates was studied to determine the effectiveness of this treatment in controlling the development of pale, soft, exudative muscle as well as improving other muscle properties. The $L\text{-}N_2$ treatment was extremely effective in preventing the development of pale, soft, exudative muscle regardless of whether subsequent equilibration was at −18 or 4°C. Most of the immersion periods lowered physiological conditions at the onset of rigor, and only the severe immersion rates showed evidence of thaw rigor.

INTRODUCTION

Physiological conditions in muscle immediately post-mortem or the temperature and pH at which rigor mortis occurs are major factors in determining the ultimate color and structure of muscle (Briskey and Wismer-Pedersen, 1961; Briskey and Sayre, 1963; Sayre and Briskey, 1963). A low pH and high temperature within the first 2 hr period post-mortem or during the onset of rigor mortis contributes directly to the development of pale, soft, exudative muscle. Although cooling isolated muscle strips at −2°C for 1 hr has been effective in preventing the development of pale, soft characteristics, it has not been possible to effectively accelerate the cooling of carcasses and thereby prevent the development of these conditions through immersion in a cold brine (Wismer-Pedersen and Briskey, 1961).

This study was made to investigate the methodology of reducing the conditions of temperature and pH immediately post-mortem and thereby prevent the development of pale, soft, exudative musculature through partial freezing with liquid nitrogen. These experiments were designed: 1) to either freeze muscle pre-rigor and avoid the consequences of thaw rigor by permitting thawing to take place in large sections (Marsh and Thompson, 1958) under normal conditions; or 2) to accelerate temperature reduction by either partial or surface freezing with liquid nitrogen.

EXPERIMENTAL

Comparison of conventional cooling, freezing, and liquid nitrogen treatment. Seventeen pigs, exsanguinated, dehaired, and eviscerated within 15 min post-mortem, were used in a preliminary trial to test the effects of post-mortem temperature reduction by various methods upon the ultimate color and structure of the muscle. The entire loin of the right side was separated into three equal sections which were subjected to one of the following temperature treatments—a) 4°C; b) −18°C; and c) −195°C—by immersion in liquid nitrogen ($L\text{-}N_2$) for periods of 20–60 sec/lb, with subsequent equilibration at −18°C. The two frozen sections were later thawed at 15°C and all three sections were evaluated for color and structure (Forrest *et al.* 1963).

Effect of liquid nitrogen treatment upon freezing depth, cooling rate and state of rigor. The second phase of this investigation was designed to study the effect of degree of $L\text{-}N_2$ treatment or immersion period upon freezing depth, cooling rate, and state of rigor.

Six pigs were slaughtered as described in the first experiment. Immediately after evisceration the right hams and lumbar loin sections were subjected to one of the following $L\text{-}N_2$ immersion periods: 5, 10, 20, 40, 60, and 120 sec/lb. The $L\text{-}N_2$ treatment was accomplished by immersing the sections in a 2-cu-ft stainless-steel tank in-

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These findings are released for public use without patent.

sulated with polyurethane foam and containing 10–12 in. of L-N_2 (−195.8°C). As the hams and loins were removed from the L-N_2 treatment they were cut, marked, and photographed to compare freezing depth.

Copper-constantan thermocouples were inserted by means of 13-gauge hypodermic needles into the biceps femoris, semimembranosus, and quadriceps of the left ham. Thermocouples were also placed in subcutaneous fat, aponeurosis, and longissimus dorsi of the left loin sections, as shown in Fig. 1. The hams and loins were subjected to the previously described L-N_2 immersion periods (excluding 120 sec/lb) and equilibrated at −18°C. Cooling rates were recorded on a Honeywell Electronik 15 Universal Multipoint recording potentiometer (temperature range −100 to +100°F). After the loins were equilibrated at −18°C the sections were cut, parallel fibril bundles were removed from the midportion (L-3) of the longissimus dorsi, and state of rigor was determined on the basis of thaw rigor changes in length and weight as described by Marsh and Thompson (1958) and Kaminer (1962). State of rigor was also determined on loin sections immersed for 120 sec/lb and a total period of 2 hr.

Effect of liquid nitrogen treatment and equilibration at —18°C upon muscle color and gross morphology. The third study was designed to determine the effects of partial freezing of prerigor muscle with L-N_2 and equilibration at −18°C upon the ultimate color, structure, pH, expressible-juice ratio, and glycogen level of the thawed muscle.

Twenty-two pigs of various breeds were held individually in a high-temperature environmental chamber at 42–45°C for periods of 20–60 min. This treatment was applied in an attempt to exaggerate post-mortem glycolytic conditions and concomitant differences in the ultimate muscle properties as described by Sayre *et al.* (1963). The carcasses were eviscerated and separated into equal sides within 15 min of exsanguination. The left sides, placed in a 4°C conventional cooler, served as controls. The untrimmed hams and untrimmed lumbar sections of the loins were separated from the right side and subjected to L-N_2 treatment as previously described. Following immersion, the sections were permitted to equilibrate at −18°C and were subsequently thawed at 15°C for comparative subjective and objective evaluation. Longissimus dorsi and biceps femoris were subjectively evaluated independently by a three-member trained panel for color and structure with the standards described by Forrest *et al.* (1963). A score of 1 represented extremely pale, soft, exudative muscle, 3 was normal, grayish-pink, and 5 was extremely dark, dry, and firm. Color of each muscle was determined with a Bausch and Lomb Spectronic 20 colorimeter with reflectance attachment at 485 mμ (Cassens *et al.*, 1963). Expressible-juice ratios were measured by the filter-paper moisture-absorption technique of Grau and Hamm (1953) as modified by Briskey *et al.* (1960). pH was determined as described by Sayre *et al.* (1963). Samples (0 and 24 hr) were frozen in a dry-ice acetone bath for determination of glycogen by the method of Dubois (1956).

Effect of liquid nitrogen treatment and equilibration at 4°C upon muscle color and gross morphology. The last phase of this investigation was designed to study the effect of short (5 sec/lb) and long (40 sec/lb) liquid nitrogen immersion

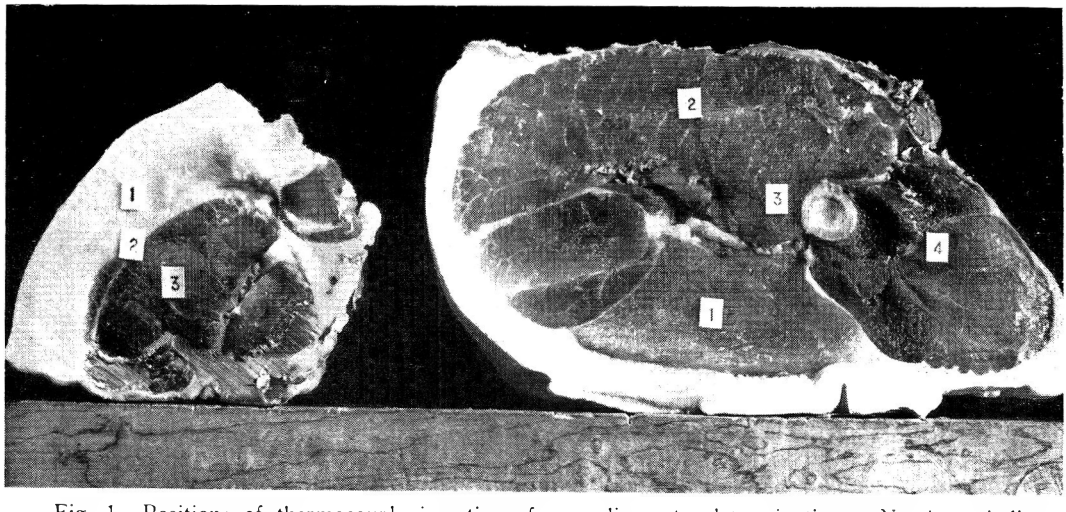


Fig. 1. Positions of thermocouple insertions for cooling-rate determinations. Numbers indicate areas of cooling-rate determinations by copper-constantan thermocouples.

periods followed by equilibration at conventional cooler temperatures of 4°C upon the ultimate color and structure of muscles. Cooling curves were obtained, and subjective and objective determinations were made as previously described.

RESULTS AND DISCUSSION

Comparison of conventional cooling, freezing, and liquid nitrogen treatment. Fig. 2 shows that L-N_2 treatment prevented

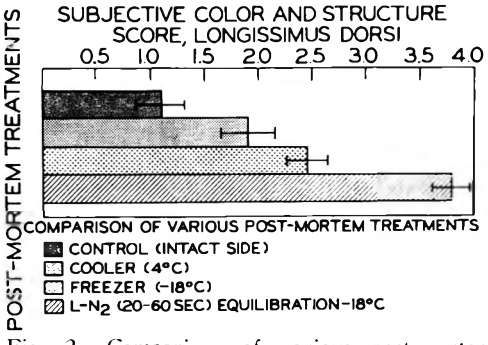


Fig. 2. Comparison of various post-mortem treatments on subjective color and structure scores. 1, extremely pale, soft, and exudative; 3, normal grayish-pink; 4, slightly dark, dry, and firm.

the development of pale, soft, exudative musculature. Although there were modest improvements by freezing at −18°C the undesirable condition was not prevented unless the temperature reduction was accelerated with liquid nitrogen treatment.

Liquid nitrogen treatment vs. freezing depth, cooling rate, and state of rigor. The immersion of ham and loin sections in liquid nitrogen for 5, 10, 40, and 60 sec/lb gave a relatively wide range in freezing depth (Fig. 3). Immersion for 120 sec/lb resulted in immediate freezing of large areas in the ham and loin, whereas the 5 sec/lb immersed sections were frozen only superficially. When the hams and loins were immersed for a total period of 2 hr the entire sections were frozen and eventually evidenced extensive fracturing of the skin, fat, and muscle. Cooling curves for the center of the longissimus dorsi (L-3) for all immersion periods with a −18°C equilibration are contained in Fig. 4. Also shown are the cooling rates for sections placed in a 4°C cooler and a −18°C freezer without L-N_2 treatment. Similarly, cooling curves for the biceps femoris (H-1) are shown in Fig. 5. The 5-sec immersion period exhibited a marked acceleration in temperature reduction during the early post-mortem period compared to conventional cooling and freezing. This acceleration in

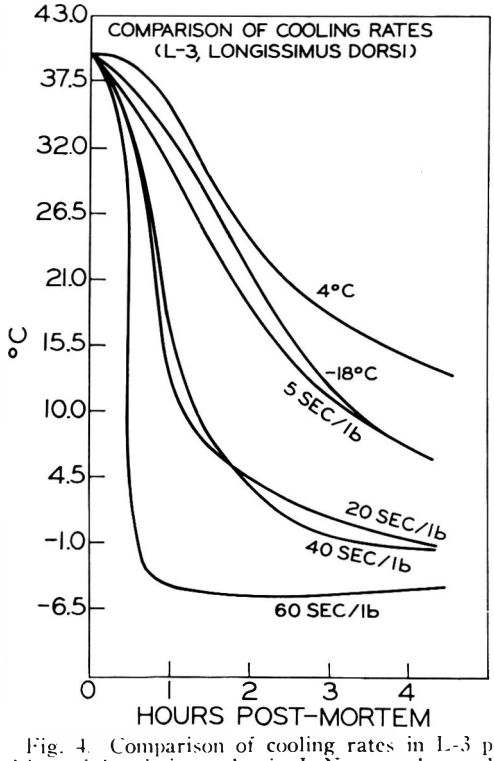


Fig. 4. Comparison of cooling rates in L-3 position of longissimus dorsi. L-N_2-treated samples for 5, 20, 40, and 60 sec/lb were equilibrated at −18°C.

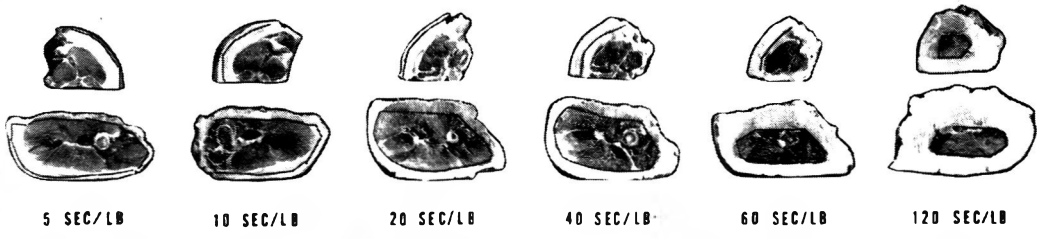


Fig. 3. Effect of various L-N_2 treatments upon freezing penetration. Black lines indicate depth of freezing.

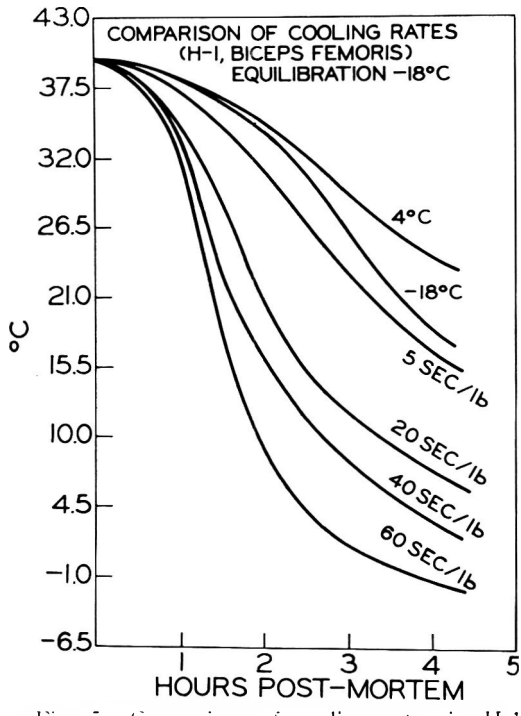
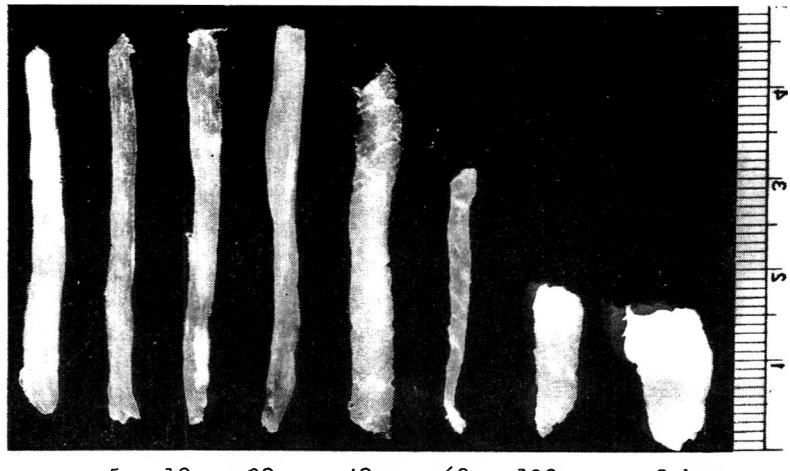


Fig. 5. Comparison of cooling rates in H-1 biceps femoris. $L\text{-}N_2$-treated samples for 5, 20, 40, and 60 sec/lb were equilibrated at −18°C.

temperature reduction was very marked for the immersion periods of 20, 40, and 60 sec. Since it is established that a combination of high temperature and low pH immediately post-mortem or during the onset of rigor mortis results in pale, soft, exudative muscle, it seems pertinent that these short immersion rates accelerated temperature reduction during these periods. Similar comparative differences were noted for all other areas indicated in Fig. 1.

The studies on the state of rigor in these muscles were conducted to determine whether, with an average post-mortem glycolytic rate, this acceleration in temperature reduction would result in freezing prior to rigor onset or whether it would force or control the development of rigor at a low temperature prior to freezing. Fig. 6 reveals that, in the sections frozen at the rates of 5, 10, 20, and 40 sec/lb, the muscles were permitted to go into rigor mortis prior to specific tissue freezing. This is evidenced by the complete absence of thaw rigor contracture as defined by Marsh and Thompson (1958). When the sections were immersed for 60 sec/lb, the muscles were apparently frozen before rigor mortis completion and showed signs of slight thaw rigor contracture. When the sections were immersed for either 120 sec/lb or a total of 2 hr the freezing occurred before the onset



Rate (sec./lb.)	C	5	10	20	40	60	120	2 hr.
% loss (length)	0	0+	0	0	2.5	17.5	52.5	57.5

Fig. 6. Determinations of state of rigor at time of freezing. All sections were approximately 4 cm in length as thaw rigor contracture experiment was initiated. Thaw rigor contracture was not significant in 5–40 sec/lb sections, slight on 60 sec/lb sections, and very marked at 120 sec and 2 hr.

of rigor and severe thaw rigor contracture was evident.

Liquid nitrogen treatment and equilibration at −18°C. It seems especially pertinent that all immersion periods prevented the development of pale, soft, exudative muscle (Fig. 7). Fig. 8 compares color and structure scores of longissimus dorsi muscles for all immersion periods with their respective groups of controls. Since a color and structure score of 1 represents extremely pale, soft musculature and 3 normal grayish pink musculature, it should be emphasized that in all cases the L-N_2 sections were normal to slightly dark regardless of the severity of the pale, soft, watery condition in their respective controls. It should be reiterated that the control and L-N_2-treated samples represent the left and right sides of the same carcass. When all carcasses were pooled, regardless of immersion rate, it was noted that, with undesirable low-quality controls, the L-N_2-treated sections were always normal. It is of further interest that when the controls were normal in color and structure the L-N_2 treatment resulted in only a slight increase in color intensity, resulting in normal to slightly dark tissue. This would appear to be a major factor since L-N_2 prevents the development of pale, soft, exudative muscle but

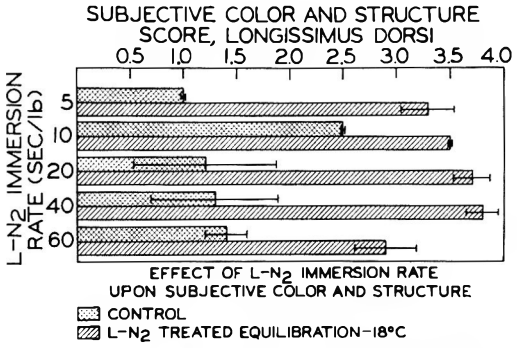


Fig. 8. L-N_2 immersion rate (equilibration −18°C) on color and structure in comparison with controls. 1, extremely pale, soft, and exudative; 3, normal, grayish-pink; 4, slightly dark, dry, and firm.

does not make normal musculature objectionably dark. Additionally, since glycogen level at the time of death may also contribute partially to the development of undesirable tissue (Briskey *et al.*, 1959; Briskey and Wismer-Pedersen, 1961), it is significant that L-N_2 treatment resulted in normal musculature regardless of whether the initial glycogen level was high or low. As can be seen in Fig. 9, the improvement in subjective color and structure was also readily detectable by objective evaluations of color and expressible juice. The improvement in expressible-juice ratio is particularly perti-

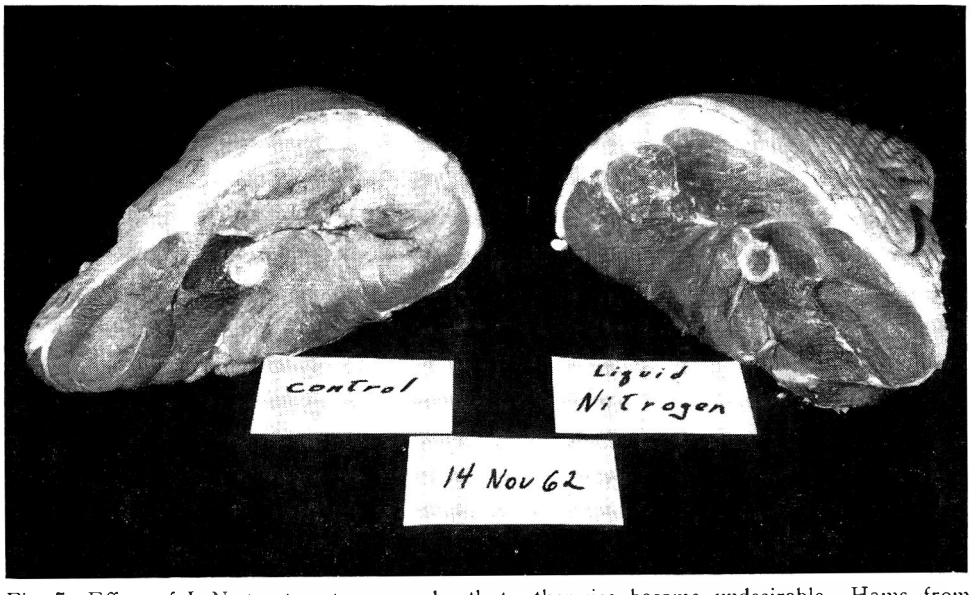


Fig. 7. Effect of L-N_2 treatment on muscles that otherwise became undesirable. Hams from the same animal. The ham on the right was treated with L-N_2; the one on the left was untreated.

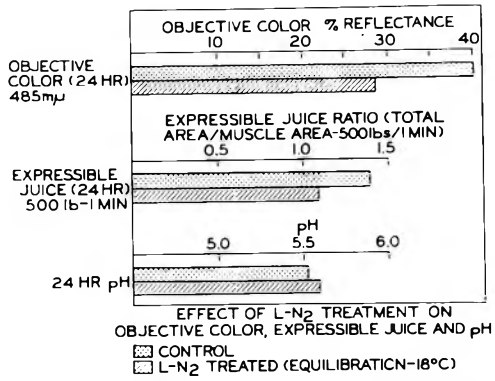


Fig. 9. Effect of $L\text{-}N_2$ treatment upon objective color, expressible-juice ratio, and pH.

nent since the $L\text{-}N_2$-treated sections were frozen and thawed and the controls were only chilled. Although further studies must be conducted on rate of pH reduction as well as ultimate pH values, there were no significant differences in 24-hr pH values between control and $L\text{-}N_2$-treated samples.

Liquid nitrogen treatment and equilibration at 4°C. Since $L\text{-}N_2$ treatment and equilibration at −18°C prevented the development of pale, soft, exudative muscle, it appeared that the usefulness of this treatment would be enhanced if the carcasses could be either sprayed with or immersed in $L\text{-}N_2$ and equilibrated at 4°C. The 4°C equilibration study included immersion periods of 5 sec/lb and 40 sec/lb because it was felt that the 5 sec/lb period approximately simulated what might be expected from spray treatment. Fig. 10 shows the cooling rate in the biceps femoris (H-1) section. It should be noted that during the initial periods the cooling rate in the 4°C-equilibrated sections was nearly identical to the cooling rates for the −18°C-equilibrated section. The 4°C-equilibrated sections, however, eventually manifested a slower cooling rate, and equilibrated before the freezing point was reached. Similar comparisons can be noted for the (L-3) position in the loin (Fig. 11). Fig. 12 illustrates the effects of 5 sec/lb and 40 sec/lb $L\text{-}N_2$ treatment and 4°C equilibration on subjective color and structure scores. The $L\text{-}N_2$ treatment produced extremely desirable muscle products although the controls were extremely pale, soft, and exudative in every case. Fig. 13 shows statistical treatment of

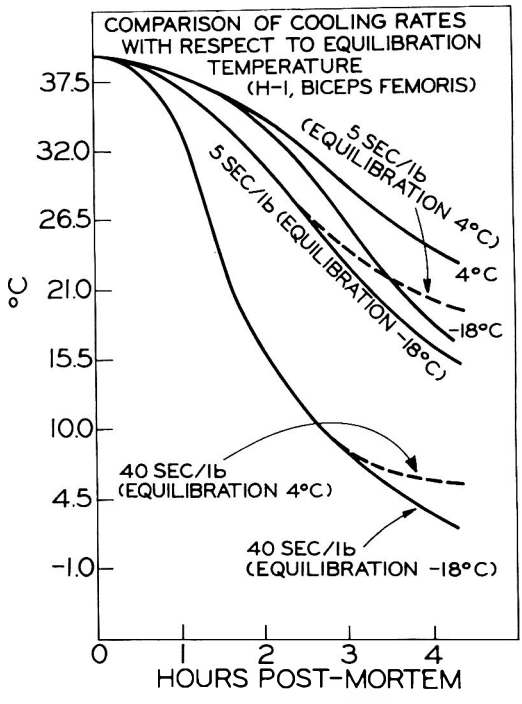


Fig. 10. Comparison of cooling rates in H-1 biceps femoris treated for 5 and 40 sec/lb and equilibrated at 4 and −18°C.

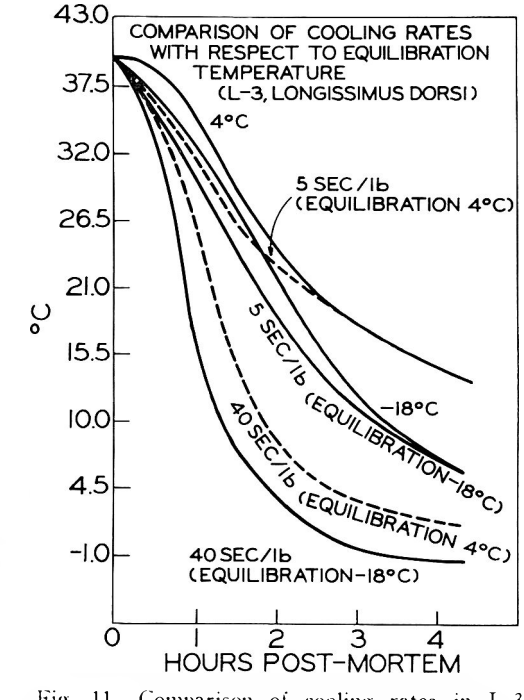


Fig. 11. Comparison of cooling rates in L-3 longissimus dorsi treated for 5 and 40 sec/lb and equilibrated at 4 and −18°C.

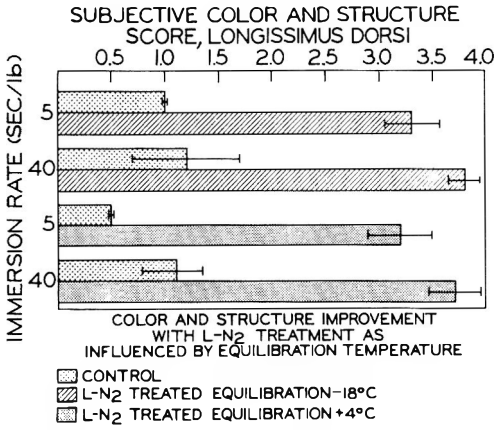


Fig. 12. Comparison of effects of immersion rate and equilibration temperatures upon muscle color and structure. 1, extremely pale, soft, and exudative; 3, normal grayish-pink; 4, slightly dark, dry, and firm.

data collected on these comparisons. The subjective color and structure scores were very highly significant (p 0.001) as analyzed by analysis of variance of paired comparisons. Muscle color, 48-hr glycogen level, and juice retention were also significantly improved (p 0.05). It might also be expected that since color and structure were preserved in the L-N_2-treated pigs they would have superior protein solubility (Sayre and Briskey, 1963). Preliminary trials with immersion periods of 60 sec/lb were also conducted with dry ice and alcohol (−70°C). This treatment was also effective in preventing pale, soft, exudative muscle. It is therefore shown that any suitable cryogenic liquid or liquefied gas over the range of −70°C to −195°C can be used effectively to prevent the development

L-N_2 TREATMENT a,b, ON L. DORSI (EQUILIBRATION 4°C)

CHARACTERISTIC c,	L-N_2	CONTROL	"T"
SUB; COLOR & STRUCTURE	3.4	0.8	9.92***
OBJECTIVE COLOR	56.3	59.6	2.80*
GLYCOGEN mg/gm	1.53	0.28	3.93*
EXP JUICE RATIO	1.85	2.23	3.10*

a AVERAGE VALUE FOR 6 PIGS
b TREATED FOR 5 AND 40 SEC/lb
c 48 HR POST-MORTEM

Fig. 13. Effect of L-N_2 treatment and equilibration at 4°C upon certain properties of longissimus dorsi. "T" test as determined by analysis of variance of paired comparisons; objective color at 485 mμ. Expressible-juice ratio as described by Sayre *et al.* (1963).

of these undesirable properties. The effectiveness of short-term immersion rates and equilibration at above-freezing temperatures point to processing-line spray applicators for liquid nitrogen or other acceptable cryogenic liquids.

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Prevention of Pale, Soft, Exudative Porcine Muscle Through Regulation of Ante-Mortem Environmental Temperature[a]

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SUMMARY

Thirty-seven Poland China pigs of uniform weight were subjected to various (warm, cold, warm-cold) ante-mortem treatments. The warm treatment (42–45°C) resulted in rapid post-mortem glycolysis, rapid onset of rigor mortis at a low pH and high temperature, and the development of extremely pale, soft, and exudative musculature with a marked loss in juice retention and protein solubility. Although muscle characteristics improved markedly from cold treatment (1–3°C), the most desirable muscles were produced by the warm treatment followed immediately by cold treatment. The latter treatment resulted in lower muscle temperature and reduced levels of glycogen and certain glycolytic components at the time of death. These muscles exhibited a limited glycolysis, rapid onset of rigor mortis but at a low temperature and high pH, and appeared dark, dry, and firm, with superior juice retention and greater protein solubility. The cold-to-warm treatment failed to provide uniformity in the development of post-mortem changes in color and structure.

INTRODUCTION

Post-mortem glycolytic rate has been shown to be a major factor in determining the ultimate color, structure, and juice retention of post-mortem musculature (Briskey and Wismer-Pedersen, 1961a; Sayre *et al.*, 1963, Sayre and Briskey, 1963). It has been further implied that when glycolysis proceeds rapidly at a high temperature with a resultant low pH, the muscle becomes pale in color intensity, soft in structure, low in protein solubility, and extremely exudative in appearance (Briskey and Wismer-Pedersen, 1961a; Briskey, 1963; Sayre and Briskey, 1963). Sayre *et al.* (1963) reported that when certain pigs were exposed to a warm environment, glycolytic rate was accelerated and adverse effects on the musculature were exaggerated. It has also been reported (Consolazis *et al.*, 1963) that an increase in metabolic activity is associated with elevated temperatures. Several workers (Wismer-Pedersen, 1959; Ludvigsen, 1954; Judge *et al.*, 1959; Osinka *et al.*, 1960) have also noted a high incidence of pale, soft, exudative tissue in pigs exsanguinated during the warm summer months. Briskey *et al.* (1960) reported wide variation in ultimate pH, color, and structure of porcine muscle during periods of major environmental change. More recently, Forrest *et al.* (1963) noted that although nearly 18% of all hams surveyed were pale, soft, and exudative, incidence of this condition was highest during periods of large daily temperature fluctuations (fall and spring). Sayre *et al.* (1961) have also noted that the subjection of pigs to a cold environment depleted initial muscle glycogen and resulted in higher ultimate pH and color intensity values. Of pertinence to this situation is the recent observation that unacclimatized animals showed a more marked response to hypothermia than acclimatized animals (Guseva, 1962).

In view of the beneficial effects of a rapid change to a cold environment and the deleterious effects of a rapid change to a warm environment, the present experiments were designed to:

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1) Determine if temperature regulation ante-mortem would control the nature of post-mortem glycolysis and thereby prevent the development of pale, soft, exudative porcine muscle.

2) Gain insight into the mechanisms by which ante-mortem temperature and temperature fluctuations contribute to levels of muscle metabolic constituents at the time of death as well as during subsequent post-mortem glycolysis.

MATERIAL AND METHODS

Experiment I. The first of two experiments in this study was conducted during August. Twelve market-weight Poland China pigs from a single herd, reared under uniform conditions, were randomly allotted to four treatment groups. Pigs in Lot I, slaughtered according to commercial practice, served as controls. Pigs in Lot II (warm) were placed in a controlled high-temperature chamber at 42–45° for 30–60 min prior to slaughter (Sayre *et al.*, 1963). Pigs in Lot III (cold) were placed in a cold-water bath (1–3°C for 30 min prior to slaughter) as described by Sayre *et al.* (1961), and pigs in Lot IV (warm to cold) were subjected first to the treatment of Lot II and then to the treatment of Lot III going from warm to cold.

The time course of rigor mortis was followed by placing a strip of muscle in a "rigorometer" within 10 min post-mortem (Briskey *et al.*, 1962). Measurements of pH, surface color reflectance, and temperatures were made at 0, ¼, 1, 2, 3, 6, and 24 hr post-mortem (Sayre *et al.*, 1963). The initial levels of glycogen were determined by the phenolsulfuric acid method of Dubois (1956). Protein solubility (24 hr) was determined by the procedures of Helander (1957). The water-binding capacity (24 hr) of the longissimus dorsi was determined by the filter-paper method described by Grau and Hamm (1953) as modified by Briskey *et al.* (1959).

Subjective color and structure scores of muscles in the ham and loin were made using the 5-point scale described by Forrest *et al.* (1963). A low score (1–2) indicated a pale, soft exudative muscle, a score of 3 was considered normal, and 5 represented dark, dry muscle.

Experiment II. The second experiment was conducted during mid-March. Twenty-five Poland China pigs of market weight (from the University herd) were allotted randomly to 5 treatment lots. Four of these treatments were identical to those described in the first experiment; however, a fifth lot was added consisting of the cold treatment followed immediately by the warm treatment.

Samples of longissimus dorsi were collected as described in the first experiment, and glycogen, pH, color, temperature, and water-binding capacity were determined as previously described.

Lactic acid was also determined at zero and 24 hr post-mortem, according to the method of Barker and Summerson (1941). The initial levels of inorganic phosphates (Pi) were determined by a slight modification of the method described by Howard and Lawrie (1956). High-energy phosphates (10′ P) were estimated by the amounts of inorganic phosphate (Pi) present after 10 min of hydrolysis (100°C in 1*N* HCl). Corrections were made for the amounts of inorganic phosphate (Pi) and creatine phosphate (CP). CP was estimated as the increase above Pi after 36 min of hydrolysis at 25°C with all reagents for phosphate determination present except molybdate. TSP was determined by digesting 5% trichloracetic acid extracts, of muscle taken at the time of death, for 10 min with 60% perchloric acid.

The data were analyzed with Duncan's new multiple-range test (Steele and Torrie, 1960).

RESULTS AND DISCUSSION

The results are subdivided by muscle characteristic, with both experiments discussed together.

Muscle temperature. Table 1 indicates that the longissimus dorsi muscle of the pig is extremely sensitive to ante-mortem environmental temperature. The treatment effect on muscle temperature at slaughter was similar for both experiments. Heat treatment produced muscle temperatures at slaughter of 45.0 and 43.4°C for Experiments I and II, respectively, whereas cold treatment resulted in muscle temperatures of about 35°C in both experiments. The warm-to-cold treatment gave muscle temperatures of 36.0 and 33.4°C at the time of death for Experiments I and II, respectively. This temperature difference of approximately 3°C may be due to the fact that the pigs in Experiment I, which were slaughtered during mid-summer, responded more rapidly to

Table 1. Treatment effect on muscle temperature.

Lot	Treatment	Muscle temperature 0 hr	
		Expt. 1	Expt. 2
I	Control	38.9	38.6
II	Warm	45.0	43.4
III	Cold	35.0	35.5
IV	Warm to cold	36.0	33.4
V	Cold to warm	.....	40.0

heat treatment and had mean temperatures 2–3°C higher (Lot II) than in Experiment II (Lot II), which were slaughtered during the spring months. Sayre *et al.* (1963) likewise conducted heat treatment during spring months, and produced muscle temperatures of only 41.7°C. Although animals in Lot V (cold, 1–3°C, to warm, 42–45°C) were held in the warm environment (42–45°C) for approximately the same period after restoring their body temperatures to normal, the muscle temperature at death never reached the elevated level observed in Lot II (42–45°C), with no previous chilling. It would appear that this difference in response to heat, in animals previously chilled and returned to normal, was not due to an insulating effect of varying degrees of external fat, since these pigs were extremely uniform, with less than 0.2 of variation in fat back thickness between all groups.

Muscle glycogen level. The glycogen level in the muscle at the time of death was very highly influenced by pre-slaughter environmental temperature. Fig. 1 shows that the warm-to-cold treatment in Lot IV (Experiment I) significantly ($p<0.05$) lowered glycogen levels below those of the control animals in Lot I. Muscle of pigs in Lot II (warm-temperature treatment) and Lot III (cold-temperature treatment) contained about one-half as much initial muscle glycogen as the controls in Lot I, although extreme variations were noted and these differences were not statistically significant ($p<0.05$). Sayre *et al.* (1961) also found that, although cold treatment apparently decreased mean glycogen levels, the results were not statistically significant ($p>0.05$). Warm-to-cold treatment (Lot IV) in Experiment II (Fig. 2) significantly ($p<0.05$) decreased muscle glycogen level below that of control pigs (Lot I). The values for Lot II (warm) and Lot III (cold) followed the same trend as in Experiment I. It seems especially pertinent that although glycogen levels were not significantly lowered by heat in these experiments or by cold treatment as previously described by Sayre *et al.* (1961), heat treatment accelerated post-mortem glycogen breakdown. Conceivably, the change from warm to cold treatment allowed accumulated lactic acid to be removed from the muscle. Although the mean glycogen level of the muscles from the pigs in Lot V (cold, 1–3°C, to warm, 42–45°C) were higher than the glycogen levels in other treatments (Fig. 2), the values were more widely scattered. This is especially interesting in view of the marked reductions in glycogen levels resulting from a reversal of these temperature treatments. It should also be noted that in general all glycogen levels of the muscles from pigs in Experiment II (spring slaughter) were slightly higher than the glycogen levels from pigs in Experiment I, which was conducted during the warm summer weather. This observation is supported by the results of other workers who report higher glycogen levels in cold-acclimatized animals (Guseva, 1962).

Lactic acid concentration at 0-hr and 24-hr periods. Lactic acid values reported in Fig. 3 were determined only in Experiment

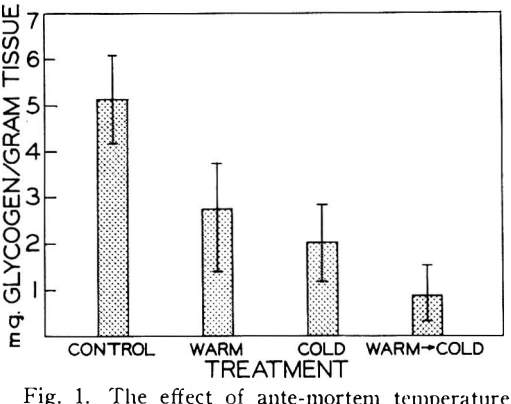

Fig. 1. The effect of ante-mortem temperature treatment on the 0-hr longissimus dorsi glycogen (Expt. I).

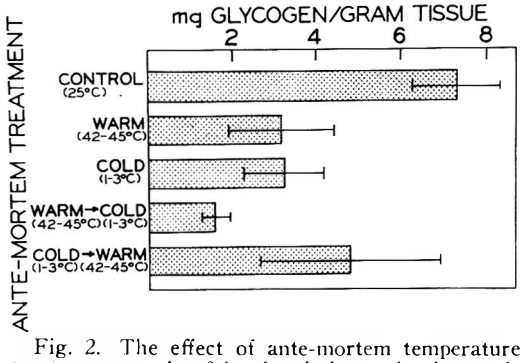

Fig. 2. The effect of ante-mortem temperature treatment on the 0-hr longissimus dorsi muscle glycogen (Expt. II).

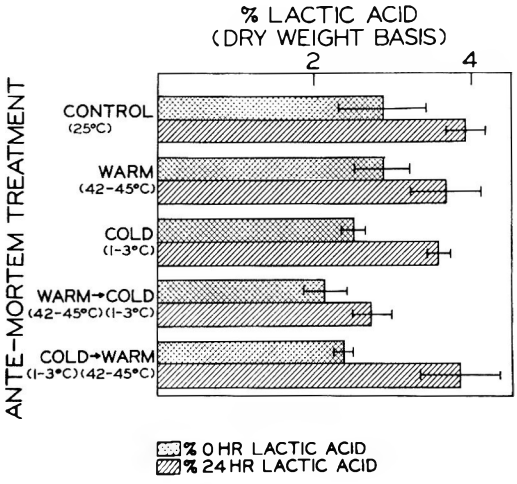

Fig. 3. The effect of ante-mortem temperature treatment on longissimus dorsi muscle lactic acid concentration (Expt. II).

II. The lactic acid values (0 hr) expressed as percentage of dry tissue were significantly lower in Lots IV (warm to cold) and V (cold to warm) ($p<0.05$) than in Lots I (control) and Lot II (warm). The heat treatment (Lot II), which has been shown to accelerate post-mortem glycolysis, apparently caused the lactic acid, produced under stress and/or anaerobic conditions, to be retained in the muscle of the live animal, with a resultant higher 0-hr lactic acid content. Conversely, when the warm pigs were placed in the cold (Lot IV), lactic acid was removed from the muscle. Several of the controls also exhibited rapid post-mortem glycolysis. This may explain the high initial lactic acid levels in Lot I (control) since Briskey and Wismer-Pedersen (1961b) have observed that pigs characterized by rapid post-mortem glycolysis also contained high levels of lactic acid in biopsy samples. At 24-hr, only Lot IV (warm to cold) was significantly ($p<0.05$) lower in lactic acid content than Lot I (control). This difference can be explained by the initial levels of muscle glycogen (Fig. 2).

Effect of treatment upon muscle phosphate. All treatments in Experiment II resulted in low creatine phosphate (CP) levels at the time of death compared to levels previously described, (Bendall *et al.*, 1964). The low levels in Lots II were probably due to the stress conditions placed on the animals prior to slaughter. The fact that these low levels of CP were not significantly different ($p<0.05$) from those of the controls was probably due to the fact that several of the controls exhibited rapid post-mortem glycolysis, which has also been shown by Bendall *et al.* (1964) to be associated with a low level of CP.

10′ P levels, an approximation of ATP, were significantly ($p<0.05$) lower in Lot IV (warm to cold) than in Lot I (control) (Fig. 4). It seems especially pertinent that none of the other environmental-temperature treatments significantly reduced the 10′ P levels. The Pi levels, however, were significantly higher ($p<0.05$) in Lots II (warm) and Lots IV (warm to cold) than in Lot I (control). Although, as will be shown later, Lot II (warm) gave a rapid post-mortem glycolysis and Lot IV a slow post-mortem glycolysis, these results are still consistent with the postulation that inorganic phosphate levels may contribute to rate of glycolysis (Krebs, 1957) since in both Lots II and IV the time course of rigor mortis was significantly ($p<0.05$) shorter. The difference in extent and rate of glycolysis between these two lots with high inorganic phosphate levels may be ascribed to the previously indicated low glycogen levels in Lots IV (warm to cold).

TSP levels were also significantly decreased in Lot IV (warm to cold) below those in Lot I (control). Although the true implications of this observation await clarification, there may have been increased

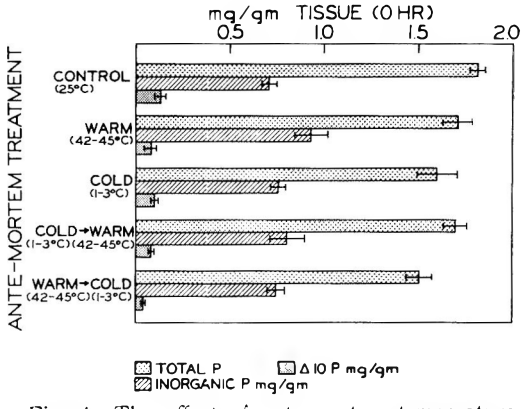

Fig. 4. The effect of ante-mortem temperature treatment on longissimus dorsi muscle phosphate (Expt. II).

phosphate excretion in Lot IV, since these animals had undergone a relatively long period of treatment. This is plausible in view of recent findings (Babayeva, 1962) of an upset of plasma ion balance in overheated rats. Except for this variation there were no differences in TSP between pigs exhibiting different rates of glycolysis, which is in agreement with the findings of Lawrie (1960) and Bendall *et al.* (1964).

Rate of glycolysis. The total time course of rigor mortis (Fig. 5) (Expt. I) was significantly shorter ($p<0.01$) in Lots II (heated) and IV (warm to cold) than in Lot I (control). The short delay phase in Lot II can be explained on the basis of the sharp drop in pH as well as the depletion of glycogen and ATP at this same period (Briskey and Wismer-Pedersen, 1961a). Conversely, the shorter delay phase in Lot IV (warm to cold) can be associated with the depleted level of glycogen (Fig. 1, 2) since Bate-Smith (1948), Bate-Smith and Bendall (1956), Bendall (1951), and Lawrie (1953) reported that as glycogen reaches a low level the muscle no longer has the capacity for sufficient resynthesis of ATP, and onset of rigor mortis occurs. This is further supported by the fact that Lot IV (warm to cold) contained a significantly lower 10′ P level (Fig. 4, Experiment II) at the time of death. Presumably the higher levels of 10′ P and glycogen in Lots I and III were responsible for regeneration of ATP, thus preventing onset of rigor mortis.

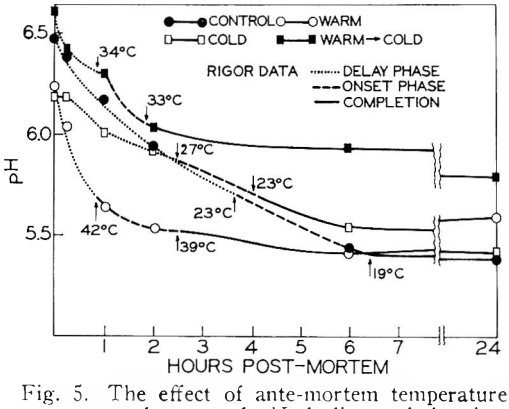

Fig. 5. The effect of ante-mortem temperature treatment on the rate of pH decline and the time course of rigor mortis. The arrowed numbers indicate mean temperatures at the onset and completion of rigor for each treatment.

It should be noted that rigor mortis onset occurred at 34°C, pH 6.3, in Lot IV (warm to cold) whereas it occurred at 42°C, pH 5.60, in Lot II (warm). Ultimate pH (24 hr) was lowest in Lots I (control) and III (cold), which was expected on the basis of the initial glycogen data. It is of further interest to note the high ultimate pH in Lot IV (warm to cold).

Fig. 6 shows that pH changes by treatment (Experiment II) follow patterns similar to those previously reported for Experiment I. Both lots I (control) and II (warm) had a rapid rate of pH decline compared with that of the other lots. The severity of pH decline was not as marked in Lot II (warm) (Expt. II) as in the corresponding Lot of Expt. I, because one pig (Expt. II) withstood the warm temperatures and metabolized its muscle glycogen and lactic acid to depletion and retained a high ultimate pH. Differences in pH between lots reached significance ($p<0.05$) at the 3- and 6-hr post-mortem periods, with Lot IV (warm to cold) being significantly higher in pH than Lot I. The 24-hr pH of Lot V (cold to warm) was also significantly higher in pH than Lot I.

Muscle color, structure, and juice retention. Fig. 7 shows the time course of the changes in surface reflectance (longissimus dorsi) (485 mμ) for the pigs in Expt. I. Lot II (warm) developed a high reflectance value (extremely pale color) rapidly after death, whereas the rate of color change in Lots III (cold) and IV (warm to cold) was markedly reduced. These reflectance

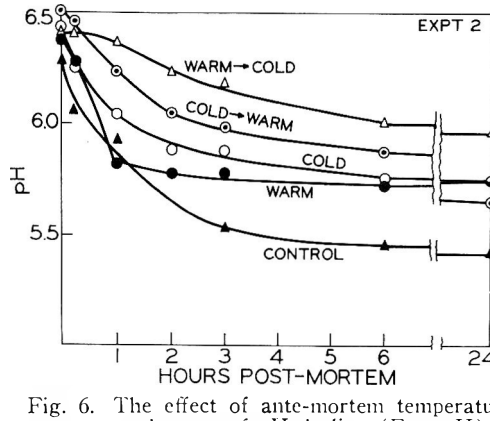

Fig. 6. The effect of ante-mortem temperature treatment on the rate of pH decline (Expt. II).

differences were also supported by subjective color and structure classification as described by Forrest *et al.* (1963). The Lot II (warm) pigs received subjective scores of 0.5 (extremely pale, soft, and watery) whereas the pigs from Lot IV (warm to cold) received subjective color scores of 3.0–4.0 (normal to dark). Color and structure differences were similar in Expt. II (Fig. 8). Subjective color scores for the biceps femoris and semimembranosus were also significantly ($p<0.05$) higher (darker and drier) in Lot IV (warm to cold) than in Lots I (control) and II (warm). Juice-retaining capacity (Fig. 9), expressed on a total area/meat film area basis (Briskey *et al.*, 1959; Sayre *et al.*, 1961), also supported the undesirability of product from pigs undergoing heat treatment (Lot II) (Sayre *et al.*, 1963). The expressible-juice ratios reflect the juice-retaining superiority of the muscles from the cold (Lot III) and warm to the cold (Lot IV) treatment.

Protein solubility. Sarcoplasmic and myofibrillar protein solubility at 24 hr post-mortem was decreased markedly in Lot II (warm) (Fig. 10). This observation can be explained on the basis of low pH and temperature at the onset of rigor and immediately post-mortem, as described by Sayre and Briskey (1963). Conversely, where rigor mortis occurred at a low temperature and high pH (Lot III, cold, and Lot IV, warm to cold) the solubilities of the proteins were high at 24-hr post-mortem. As Cassens *et al.* (1963) observed by electron microscopy, the muscles in Lot IV (warm to cold) that have high protein solubility also maintained a high degree of ultrastructure integrity, whereas the muscle from the warm treatment (Lot II) became disrupted and slightly granular.

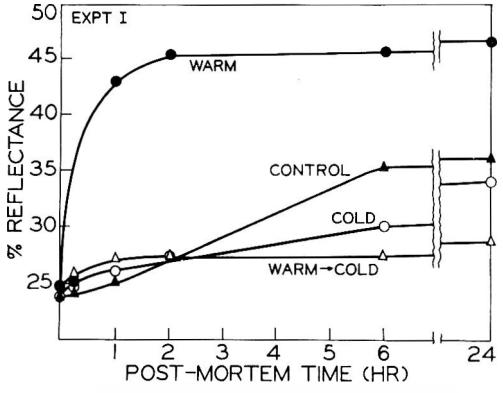

Fig. 7. The effect of temperature treatment on post-mortem muscle reflectance color changes as determined by a reflectance attachment on a Bausch and Lomb Spectronic 20 colorimeter.

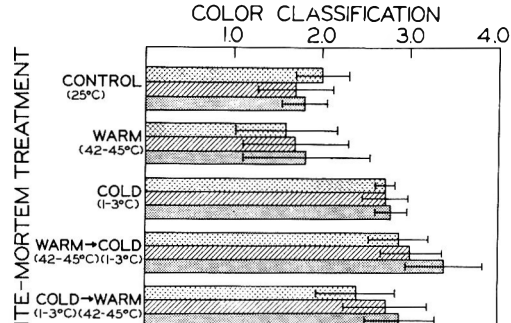

Fig 8. The effect of ante-mortem treatment upon ultimate muscle color classification. Based on a 5-point scale. A low score indicates a pale, light color, and a high score indicates a dark color.

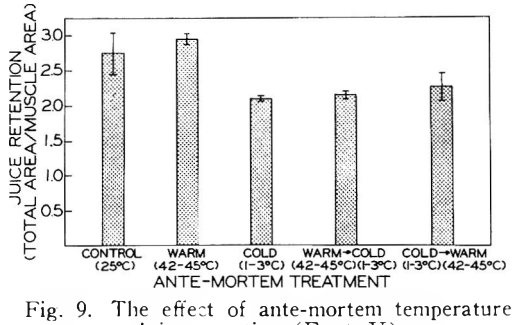

Fig. 9. The effect of ante-mortem temperature treatment on juice retention (Expt. II).

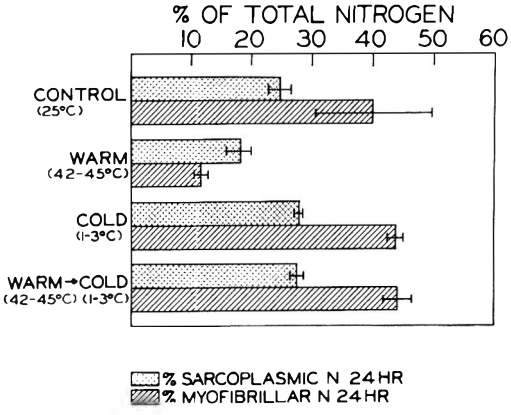

Fig. 10. The effect of ante-mortem temperature treatment on protein solubility (Expt. I).

ABBREVIATIONS USED IN THIS PAPER

P_i = Inorganic phosphate
CP = Creatine phosphate
10′ P = An estimate of ATP
ATP = Adenosine triphosphate
TSP = Total soluble phosphate
—|— on graphs is standard error of the mean

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Development of Microorganisms and Fluorescence on Poultry Dipped in Water Containing Iron [a]

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SUMMARY

Studies were undertaken to determine the effects of iron on the microbiological quality and fluorescence of poultry dipped in solutions containing different concentrations of the metal. When pseudomonads were grown in different broth media, fluorescent pigment production differed with bacterial species, amount of iron, and medium used. Growth was inhibited by the chelating agent, 8-hydroxyquinoline; inhibition was reversed by addition of iron. When chicken was dipped in solutions containing 0, 0.1, 1, and 5 ppm iron, and stored 1 week at 5°C, fluorescence was greatest in broth inoculated with organisms from poultry treated with the two highest concentrations. Bacterial growth was also greatest on chicken in the presence of 1 ppm added iron.

The effect of iron in increasing the rate and extent of bacterial spoilage in shell eggs has been emphasized by Garibaldi and Bayne (1960, 1962a,b). Iron reverses the action of conalbumin in controlling the growth of *Pseudomonas* and other gram-negative bacteria in egg white (Schade and Caroline, 1944; Feeney and Nagy, 1952; Garibaldi, 1960). However, stimulation of bacterial growth in eggs by iron is a result not merely of saturation of conalbumin, but also of availability of increased amounts of the essential nutrient (Garibaldi and Bayne, 1962a,b). Board (1962) found that only a fraction of the amount of iron needed to saturate conalbumin enhanced multiplication of *Pseudomonas fluorescens* in shell membranes. Considering these findings, it is not unexpected that iron may stimulate the growth of fluorescent pigment-producing pseudomonads on poultry meat (Kraft and Ayres, 1961). The present study was undertaken to evaluate the effects of different concentrations of iron on the microbiological quality of processed poultry dipped in solutions containing the metal. Investigated in addition was the relation of removal of iron by combination with a chelating agent to the growth of pseudomonads.

MATERIALS AND METHODS

Cultures of *Pseudomonas aeruginosa* (2F41) (Dept. of Bacteriology, Iowa State University, Ames), *P. fluorescens* (strain K, isolated from spoiled chicken), and *P. polycolor* (PP 2) (Dept. of Bacteriology, University of California, Davis) were grown in different media designed to enhance pigment production. Sterile solutions of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) were added to asparagine broth (Georgia and Poe, 1931), to a medium containing glycine and leucine (Burton *et al.*, 1948), and to the ammonium phosphate medium of Paton (1959). The concentrations of iron tested were 0, 0.05, 0.1, 0.2, and 0.4 ppm. Flasks containing inoculated media were shaken 18–20 hr at 15°C and incubated without shaking for an additional 18–20 hr. The broths were then centrifuged, and fluorescence measurements were made of the supernatant liquid by using a Coleman Model C photofluorometer with B_1 and PC_2 filters and 6 ppm fluorescein as standard. pH was determined with a Beckman Zeromatic pH meter.

The effect of removal of iron on the growth of pseudomonads was tested, with 8-hydroxyquinoline (oxine) used to bind the metal. A stock solution was made of 35 mg oxine in 200 ml demineralized water. Double-strength nutrient

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broth (Difco) was prepared, and traces of iron were removed by the method of Waring and Werkman (1942). The medium was diluted to normal strength with oxine and/or iron solutions and inoculated with one of the following test organisms: *P. taetrolens* (Dept. of Dairy and Food Industry, Iowa State University, Ames), *P. aeruginosa* (2F41) (Dept. of Bacteriology, Iowa State University, Ames), and *P. fluorescens* and *P. oleovorans* (supplied by Dr. W. L. Gaby, Dept. of Microbiology, Hahnemann Medical College and Hospital, Philadelphia, Pennsylvania). After incubation for about 36 hr, turbidity was measured with a Bausch and Lomb Spectronic 20 colorimeter set at 660 mμ. Pour plates were also made from the broths into trypticase soy agar (BBL) to estimate growth.

Iron analyses were performed by a procedure described by Uzumasa and Nishimura (1955) and the 1,10-phenanthroline method (American Public Health Association, 1960).

Freshly cut chicken wings purchased at a retail store were dipped in solutions containing 0, 0.1, 1.0, and 5.0 ppm iron. The poultry was allowed to drain and was then packaged in 195 LSAD cellophane. Packages were stored in a display case at approximately 5°C. The case was defrosted twice daily, when the temperature increased to 10°C, but in no instance did it remain above 5°C for more than 1 hr. Samples were examined at intervals during a period of one week for numbers of microorganisms and for fluorescent pigment production. Surfaces of wings were sampled by firmly rolling moist absorbent cotton swabs over two 2-sq-cm areas outlined by sterile metal guides. Total counts were made from pour plates of trypticase soy agar (BBL). For determinations of fluorescing bacteria, a surface plating technique (Silliker *et al.*, 1958) was employed with Medium B of King *et al.* (1954). Plates for total counts and fluorescing organisms were incubated at 15°C for 3–4 days. Numbers of coliform bacteria and enterococci were estimated by plate count, respectively using Difco violet red bile and KF streptococcus agars. These plates were incubated 24–36 hr at 37°C. Fluorescence measurements were made of asparagine broth inoculated with a swab from the poultry and then treated in a manner similar to that previously described for determination of fluorescent pigment production. Chicken wings were also examined for fluorescence under ultraviolet light.

RESULTS AND DISCUSSION

Fig. 1 illustrates that in asparagine broth an inverse relation was observed between iron content and fluorescence produced by either *P. aeruginosa* or *P. polycolor*. These results are in agreement with those of Totter and Moseley (1953) for a similar medium and range of iron concentrations. Iron content had little effect on amount of pigment elaborated by *P. fluorescens* in asparagine broth. This medium provided sufficient iron and other nutrients to satisfy the requirements of *P. fluorescens*, thereby suppressing synthesis of fluorescent pigments. However, in a medium containing glycine and leucine (Fig. 2), the fluorescent pigment developed by all organisms tested was greater with 0.05 or 0.1 ppm added iron than with 0, 0.2, or 0.4 ppm. Results were similar for *P. polycolor* in the ammonium phosphate medium described by Paton (1959); the amount of fluorescence produced was greater in broth containing 0.05 ppm added iron than in media containing higher levels or no added iron. Nitrogen source, salt composition of the medium, and the strain of bacteria appeared to influence pigment production in the presence of different amounts of iron. For *P. aeruginosa* and *P. polycolor*, pigment production in asparagine broth was also affected by increasing iron concentration. In other media, when the concentration of added iron was 0 ppm, growth of

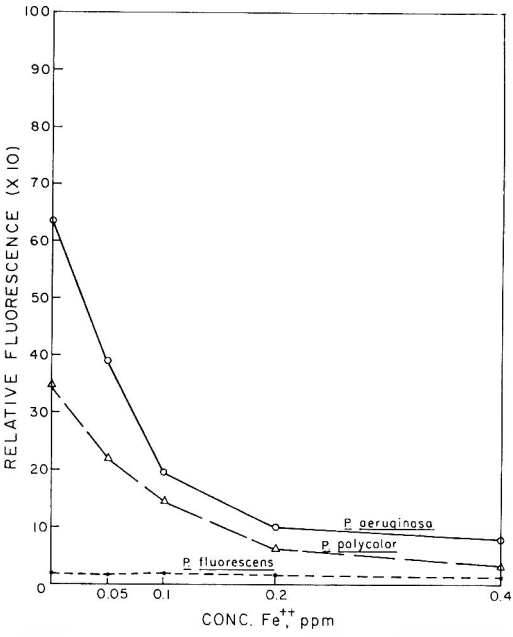

Fig. 1. Relation of iron content of asparagine broth to fluorescence produced by pseudomonads.

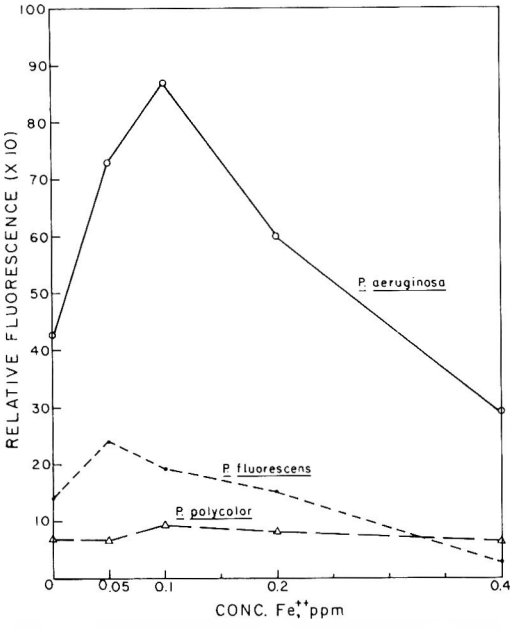

Fig. 2. Relation of iron content to fluorescence produced by pseudomonads in a medium containing glycine and leucine.

fluorescing organisms was limited. The level of contaminating iron was less than 0.1 ppm in all media before addition of the metal.

Totter and Moseley (1953) indicated that fluorescent pigment might function in a manner similar to that of non-fluorescent pyocyanine as a respiratory pigment substituting for cytochrome. With increased iron for combination with the pigment, more respiratory activity may be supplied by the fluorescent pigment pyoverdine. As pyoverdine is utilized, less pigment is elaborated into the medium (Knight, 1951). In the absence of iron the pigment is useless and is excreted. Also, it is possible that addition of iron may increase production of cytochromes, thereby reducing the need for the accessory respiratory pigment. Pseudomonads have a high iron requirement; according to Waring and Werkman (1943), *P. aeruginosa* required 3–4 times as much iron (0.075–0.100 ppm) as did other heterotrophs for maximal growth. In the present study, pyoverdine formation and growth were related. By the time that pigment became apparent in the medium, counts of all organisms were usually in excess of one million cells per ml. This might indicate that elaboration of the pigment into the medium did not occur until all the available iron had been utilized by the organisms. The bacteria were then growing in an "iron-free" medium, a condition essential for pigment excretion.

When chicken wings were examined under ultraviolet light, localized fluorescence was evident after 2–5 days of storage. Fluorescence developed initially at the edges of the wings, but at the end of 7 days was widespread over the surface of the skin. As reported previously (Kraft and Ayres, 1961), counts of fluorescing organisms were about 1,000,000 per cm^2 when fluorescence was noted on the skin. Little difference was observed in the amount of fluorescence visible on the poultry, regardless of iron treatment. However, of the iron concentrations tested, the greatest amount of pyoverdine was produced in broth inoculated with swabs from chicken treated with 1 and 5 ppm iron.

Fig 3 shows the regression of photofluorometer readings on numbers of fluorescing bacteria recovered from chicken

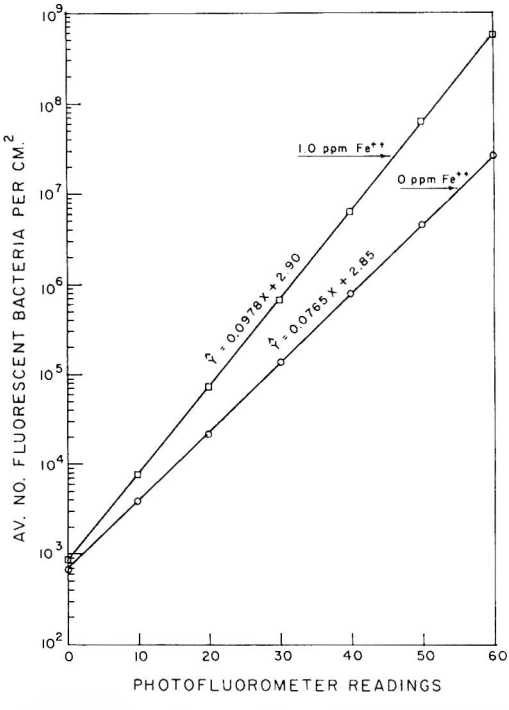

Fig. 3. Relation of photofluorometer readings to numbers of fluorescent bacteria recovered from chicken.

treated with 0 and 1 ppm iron. As may be expected, a linear relation existed between counts of pseudomonads and amount of fluorescent pigment produced. Also, for a given amount of fluorescence, bacterial counts were greater when 1 ppm iron was added to the dip solution than when no iron was added. Regression lines for 0.1 and 5 ppm iron (not shown) were intermediate to the two curves on the graph, indicating that 1 ppm was more favorable for bacterial growth than the higher level of 5 ppm. Figs. 4–7 present further information relating iron concentration in the dip solutions to the development of various organisms on poultry. Total numbers of aerobic bacteria, fluorescing organisms, and enterococci on chicken were generally greater for 1 ppm iron than for 0, 0.1, or 5 ppm iron. Stimulation of growth of total numbers of aerobes by 1 ppm iron was most pronounced during the early part of the storage period (Fig. 4). For fluorescing bacteria, all concentrations of iron tested produced a shorter lag period than observed when no iron was added to the dip (Fig. 5). Growth of coliforms appeared to be enhanced by iron, although differences between effects of various levels of the metal were not great (Fig. 6). Enterococci demonstrated greater decrease in numbers during the lag phase when iron was not added than when it was (Fig. 7). Table 1 shows changes in pH of the asparagine broth used for measurements of fluorescence. In general, pH values were greatest for media inoculated with swabs from chicken having the highest bacterial counts throughout the storage period. Increases in pH were associated with the development of fluorescing organisms and with increases in fluorescence values.

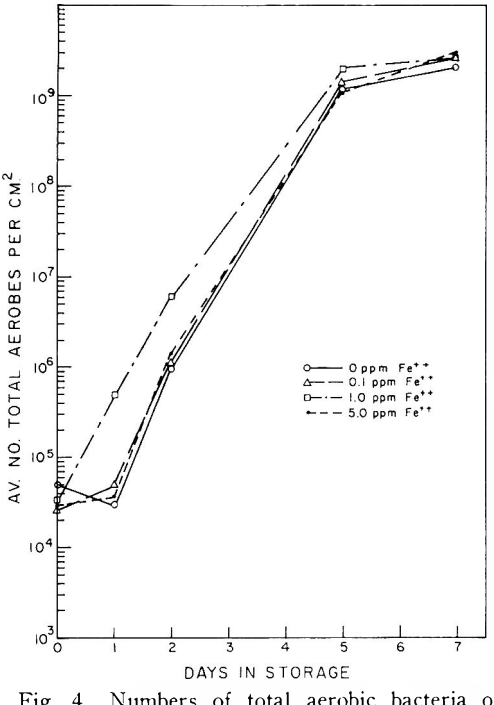

Fig. 4. Numbers of total aerobic bacteria on chicken dipped in solutions containing different iron concentrations and stored 1 week at 5°C.

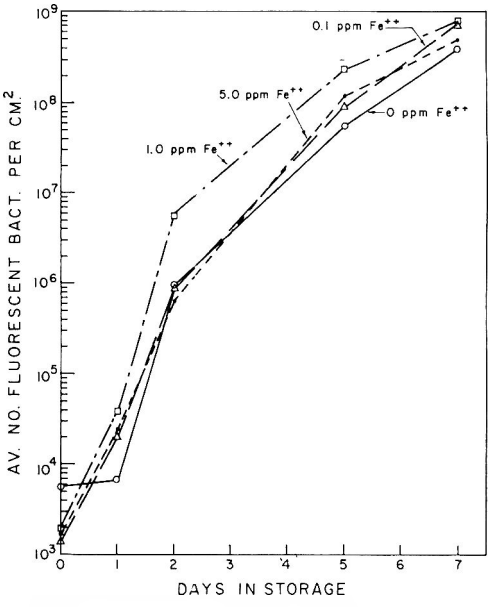

Fig. 5. Numbers of fluorescent organisms on chicken dipped in solutions containing different iron concentrations and stored 1 week at 5°C.

Table 1. pH values of asparagine broth inoculated with organisms from chicken stored at 5°C after dipping in 0, 0.1, 1, and 5 ppm iron.

Conc. iron in dip solution (ppm)	Days in storage				
	0	1	2	5	7
0	7.2	7.5	7.9	8.2	8.4
0.1	7.4	7.6	7.9	8.2	8.5
1	7.3	7.9	8.2	8.5	8.5
5	7.8	7.7	8.1	8.3	8.5

The complex nature of mineral requirements for bacterial growth has been reviewed by Knight (1951). Metals that

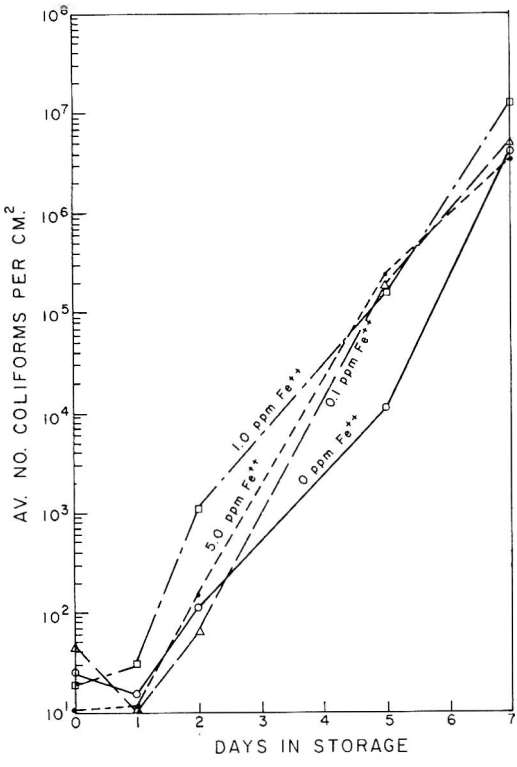

Fig. 6. Coliform counts on chicken dipped in solutions containing different iron concentrations and stored 1 week at 5°C.

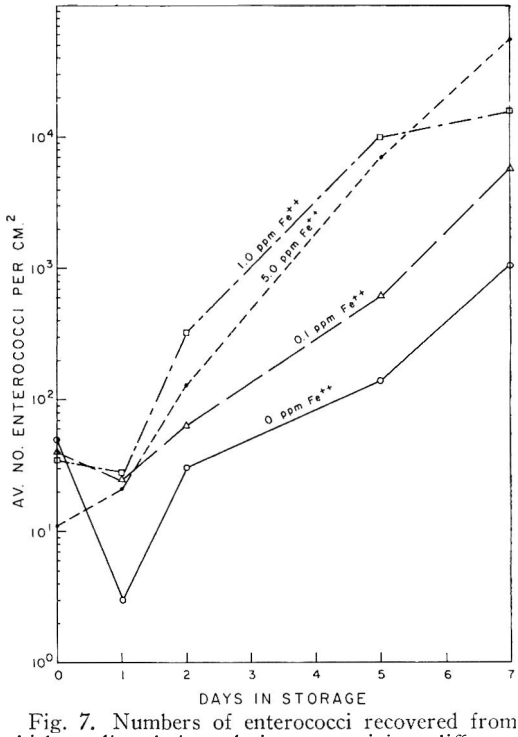

Fig. 7. Numbers of enterococci recovered from chicken dipped in solutions containing different iron concentrations and stored 1 week at 5°C.

precipitate because of changes in the pH of the medium may become unavailable or even inhibitory if they become adsorbed on cell surfaces. Precipitated iron was considered by Waring and Werkman (1942) to be injurious because of adsorption. In the present work, the relatively high level of 5 ppm iron may have exerted an inhibitory effect by adsorption, whereas the concentration of 1 ppm may have favored growth of microorganisms by providing additional nutrient. Although these studies indicated that an initial stimulus to bacterial growth may result during the storage of poultry previously in contact with small amounts of iron in solution, it is unlikely that such an effect would persist after the available iron has been utilized. By the end of the 7-day storage period, populations of total aerobes and fluorescing bacteria reached high levels (Figs. 5 and 6) regardless of iron treatment. To obtain a more complete picture of the effects of iron in water absorbed into poultry meat and adsorbed on the skin surfaces, other investigations are being directed toward evaluation of prolonged holding of poultry in chill solutions containing various amounts of the metal.

Table 2 shows the relation of the chelation of iron by oxine to the growth of several pseudomonads. All organisms were inhibited by the oxine; absorbancy readings were lowest when oxine was present in the medium. For all species of *Pseudomonas* tested, media containing oxine with 1 ppm added iron showed greater turbidity than did broth with oxine and no iron added. Inhibition by oxine was suppressed to some extent by the addition of iron. The antimicrobial activity of oxine has been studied by Albert *et al.* (1953) and reviewed by Weinberg (1957). The former authors reported that when trace amounts of Fe^{++} were absent, as in the present work, oxine was toxic to gram-negative organisms. Toxicity was decreased by adding iron. The findings reported here are in agreement with those of Albert *et al.* However, it should be emphasized that biologically im-

Table 2. Effect of iron and oxine on growth of pseudomonads.

	Absorbancy at 660 mμ				
	Control no Fe^{++}, no oxine	Oxine [a] no Fe^{++}	0.1 ppm Fe^{++}	1 ppm Fe^{++} + oxine	1 ppm Fe^{++} no oxine
P. tactrolens	0.149	0.012	0.145	0.066	0.116
P. aeruginosa	0.223	0.016	0.269	0.070	0.301
P. fluorescens	0.146	0.069	0.144	0.118	0.153
P. oleovorans	0.157	0.066	0.133	0.116	0.147
Av. all organisms	0.168	0.041	0.173	0.092	0.190

[a] Oxine concentration = 35 mg in 200 ml demineralized water.

portant metals other than iron may also form complexes with oxine, and this may result in additional growth inhibition. Also, the relationships of iron and metal-binding agents to bacterial growth on poultry meat have not been investigated; such studies are now in progress.

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RESEARCH NOTE

Changes in Some B Vitamins During Molding of Soybeans by Rhizopus Oryzae in the Production of Tempeh Kedelee

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Tempeh kedelee is an important Eastern-Asiatic food, made by allowing *Rhizopus oryzae* (and accompanying yeasts and bacteria) to grow for 2–3 days at tropical temperatures on the cotyledons of the soaked, cooked, and peeled soybeans. The hyphae penetrate between the cells in the outermost layer, and secrete enzymes that penetrate further. As a result of the enzymatic action on the cell walls, the cells in tempeh separate easily when the cotyledons are either masticated, squeezed, or shaken with water.

The digestibility of the cotyledons is greatly increased over that of merely cooked ones, probably as a result of both this physical change and a predigestion of cell wall substances, proteins, and fats by the fungal enzymes. Part of these chemical changes have been studied by Boorsma (1900), Van Veen and Schaefer (1950), Steinkraus *et al.* (1960), and Wagenknecht *et al.* (1961).

Our knowledge of the vitamin contents in tempeh is very limited. Jansen and Donath (1924) concluded from experiments with rats that the vitamin-A content was about the same as in raw soybeans.

Using a pigeon test, Jansen (1923) and Van Veen (quoted by Van Veen and Schaefer, 1950) found considerable decreases in thiamin. It remained unknown whether the loss had occurred during cooking or during fermentation.

Since we wanted to know whether the tempeh fungus increases or reduces the content of thiamin and of riboflavin, tempeh samples and the corresponding cooked and inoculated soybeans were analyzed. The fermentation was carried out both with a pure culture of the fungus on sterile cotyledons and according to the normal procedure, which allows a limited growth of other organisms. With the pure-culture tempeh, the change in niacin content was determined in addition.

PROCEDURE

Tempeh preparation. Our procedure differed slightly from that described by Steinkraus *et al.* (1960). After soaking overnight in 1% lactic acid and boiling for 10 min, the steep water was drained off and the beans were peeled by kneading and rubbing with a tennis ball in a big mortar or in a dish (in Indonesia, peeling is done either before or after steaming or cooking, by treading the drained beans in a plaited bamboo basket with bare feet).

Then the skins were removed by flotation, as is done in Asia, *viz.* by placing portions of the mass in a shallow basin-shaped sieve that is immersed repeatedly in a tub filled with water and thereby moved also in a horizontal direction in such a way that the skins float off and sink. For large-scale production it is recommended to remove the skins before soaking.

The cotyledons were spread on the trays in a drying cabinet at 60°C and frequently stirred, until they were superficially dry (water on wet basis decreasing from 64 to about 55%). In the senior author's experience both in Japanese P.O.W. camps in Indonesia and in the present laboratory, superficial drying gives the mold a great advantage over the bacteria at the start of the molding process, and this lead is retained for a longer time. In Indonesia sun drying is usually applied.

Inoculation was carried out by mixing with a small quantity of dried and powdered sporulated tempeh. Then, flat plastic boxes of 10 × 20 × 3 cm with good-fitting lids, as are used in ice-chests, and provided with six holes of 2 mm diameter in the bottoms and the lids, were filled with occasional shaking, closed and piled up in a thermostat of 27°C. After 24 hr, all boxes were turned upside down and left for another 24 hr in experiment I (optimal quality) and for 48 hr in experiment II. (Bigger quantities may be produced by spreading 3 cm thick on any kind of perforated big trays that are stacked and covered with gunny

Table 1. Changes in thiamin, riboflavin, and niacin in cooked soybean cotyledons during molding by *Rhizopus oryzae*.

Expt. no.	Molding	Ash (mg per g dry pwdr.)	Vitamin-content (μg/g dry powder or ash)					
			Thiamin		Riboflavin		Niacin + n.amid	
			Pwdr.	Ash	Pwdr.	Ash	Pwdr.	Ash
I	before	36	5.5	153	3.4	94		
	48 hr	38	3.8	100	10	270		
II	before	30			3.0	100		
	72 hr	34			17	500		
III	before	42	7.8	188	2.6	62	17.5	425
(pure culture)	72 hr	45	5.8	130	8.6	193	65	1450

bags in such a way that aeration in all regions is kept between proper limits, supplying enough air without desiccating the mass, and provided the temperature nowhere exceeds 40–45°C.)

Analyses. Riboflavin was determined fluorometrically according to the official method of the AOAC (1960), described under nos. 39033–39036 (beans: 39035a, tempeh: 39035b). However, instead of 20 mg dithionite, 1.3 ml conc. hydrochloric acid was added for eliminating the riboflavin fluorescence.

Thiamine was also determined fluorometrically according to the official method AOAC (1960), nos. 39018–39024. Takadiastase was used for enzymatic hydrolysis, and Decalso was purified according to 39018e.

Niacin and niacinamide were determined with the titrimetric microbiological method of the AOAC (1960), nos. 39066–39070, using Difco culture media in the preparation of the media mentioned in 39042 and 39043 and *Lactobacillus plantarum* ATCC no. 8014 as the test organism.

All determinations were in triplicate. The results were expressed both per g of dry powder and per g of ash. The latter data reflect more exactly the actual change in the vitamins during the process of molding, since the ash substances obviously cannot change in absolute amount. The dry weight, however, decreases about 7%. Ash was determined with the official method AOAC (1960), no. 13.071 (22010).

RESULTS AND DISCUSSION

The results are represented in Table 1.

Because of dry-weight losses of apparently 5–12%, ash content decreases 0.2–0.4% in the different experiments.

The conclusions regarding the changes in thiamin and riboflavin as a result of the molding are the same in qualitative respect, based on changes in the contents either per g dry weight or per g ash. There also seem to be no major differences between normal impure tempeh fermentation and pure-culture fermentation.

Of the thiamin present in the cooked cotyledons, about one-third was used up by the fungus. Riboflavin and niacin, however, increase considerably.

Although a great many fungi are known to require either thiamine or its building blocks, the thiamin decrease is contrary to expectation, since *Rhizopus oryzae* is autotrophic in this respect and its growth may even be inhibited by addition of thiamin to the culture medium (Schopfer, 1935; Robbins and Kavanagh, 1942). The results with riboflavin and niacin are in line with the fact that, with one exception, all fungi tested so far, including *Rhizopus oryzae*, are autotrophic with respect to riboflavin and nearly all fungi with respect to niacin (see, e.g., Fries, 1961; Peltier and Borchers, 1947).

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Biological Evaluation of Protein in Steamed and Baked Breads and in Bread Ingredients

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SUMMARY

The protein quality of baked whole-wheat bread and of the unbaked ingredients was estimated from: 1) change in body weight of rats; 2) protein efficiency ratio; 3) biological value (nitrogen-balance method); and 4) net protein utilization (change in carcass nitrogen). Steamed bread was evaluated by methods 1, 2, and 4. By all methods, the protein quality of baked bread was significantly less than that of the unbaked ingredients. Differences ranged from 9 to 53%, depending on the method of testing. The protein quality of steamed bread was the same as, or slightly less (0 to 9%) than, that of the unbaked ingredients.

That the quality of the protein in wheat flour mixtures decreases upon baking has been demonstrated in bread (Sabiston and Kennedy, 1957) and in other baked wheat mixtures (Block *et al.*, 1946; Kennedy and Sabiston, 1960). In these studies the sole criterion of evaluation was protein efficiency ratio. Losses ranged from 11 to 33%. Rosenberg and Rohdenburg (1951) showed that total lysine, as determined by microbiological assay, decreased 2–16% (av. 11%) when bread dough was baked.

In the present study, the quality of the protein in whole-wheat bread ingredients and in the baked bread was determined by several biological methods, including growth rate of rats, protein efficiency ratio, net protein utilization as shown by carcass analysis, and biological value. Steamed bread was included in some of the experiments to determine the effect of the absence of crust on protein quality.

MATERIALS AND METHODS

Materials. Whole-wheat bread was made from the following formula (in parts): whole-wheat flour (2.66% nitrogen), 100; salt, 2; hydrogenated vegetable shortening, 2; sucrose, 2; and active dry yeast, 4. Doughs were fermented 75 min at 28°C (first punch after 45 min), scaled to 22-oz loaves, and proofed for 55 min. For steamed bread, the loaves were cooked for 20 min in an institution steamer. For baked bread, the loaves were baked 25 min at 218°C.

Both the steamed and the baked breads were sliced and air-dried at room temperature for about 48 hr. The dried slices were finely ground for use in the various diets.

Diets. Three diets were prepared, from the unbaked bread ingredients, the steamed bread, and the baked bread. Composition of the diets is given in Table 1. A factor of 5.83 was used to convert nitrogen in the whole-wheat flour and bread to protein. Before the diet containing the unbaked ingredients was mixed, the active dry yeast was heated 30 min at 82°C to kill the yeast. Used in determining net protein utilization and biological value was a low-protein egg diet containing 4.5% egg protein. The egg diet contained 8% whole dried egg, 2% salt mixture (Hubbell *et al.*, 1937), 3% confectioner's sugar, 79% cornstarch, and 8% hydrogenated vegetable shortening.

Animal experiments. The protein quality of the breads was evaluated by: growth of rats, protein efficiency ratio, net protein utilization, and biological value.

Rats of the Long-Evans strain, from the stock colony of the Department of Nutritional Sciences, were weaned at 21 days of age, at which time they weighed 45–50 g. After weaning, the animals were caged separately and fed a starter diet for two or three days until they became accustomed to their new environment. That diet was composed of 6% vitamin-free casein, 80% confectioner's sugar, 10% hydrogenated vegetable shortening, and 4% USP No. XIV salt mixture. All experimental animals were given a vitamin supplement three times weekly (calculated per day: thiamine HCl, 43 μg; riboflavin, 43 μg; niacin, 171 μg; biotin, 8.6 μg; folic acid, 8.6 μg; menadione, 50 μg;

Table 1. Composition of diets (%).

Protein source	Nitrogen in protein source	Diets				
		Nitrogen	Protein source	Salt mix[a]	Confectioner's sugar	Added fat[b]
Unbaked bread ingredients	2.50	2.20	90.0	2.0	0	8.0
Steamed bread	2.61	2.21	85.7	2.0	4.3	8.0
Baked bread	2.60	2.21	85.7	2.0	4.3	8.0

[a] Hubbell, Mendel and Wakeman (1937).
[b] Primex hydrogenated vegetable shortening.

vitamin B_{12}, 0.2 μg; pyridoxine HCl, 62 μg; calcium pantothenate, 171 μg; vitamin A, 100 IU; viosterol, 10 IU; alpha-tocopherol, 0.5 mg).

Growth and protein efficiency ratio. The rat-growth method of Osborne *et al.* (1919), with modifications, was used for determination of protein efficiency ratio (PER). The average initial weight of the animals was 51 g. Three groups of 10 animals each, equally distributed as to litter, sex, and weight, were each placed on one of the three test diets and fed *ad libitum* for 28 days. Weights and food intakes were recorded.

Net protein utilization. Net protein utilization (NPU) was determined according to the method of Bender and Miller (1953a) and Miller and Bender (1955). In this experiment, animals were weaned at 31 days of age, to ensure body weights of 45–50 g. Four litters of 6–8 rats each were divided into four groups of four each. Each group contained one rat from each litter, and the total weights of each group were as nearly equal as possible. The average initial body weight of the groups was 50 g in the first experiment and 47 g in a duplicate experiment. All animals were fed the starter diet for two or three days, after which three groups were fed the three different test diets, and the fourth group was given a 4.5% egg protein diet. At the end of 10 days' feeding, the animals were fasted for 8 hr and sacrificed by gassing. Each animal was weighed immediately before and immediately after sacrifice, so that both PER and NPU could be calculated. Each carcass was placed in a sealed plastic bag and frozen until analyzed. Carcasses were dried in a vacuum oven for 48 hr at 64°C for moisture determinations according to the procedure given by Bender and Miller (1953b). The dried carcasses were extracted with ethyl ether for 44 hr. The dry, fat-free carcasses were then ground in a hand grinder and pulverized with a mortar and pestle, to produce a homogeneous mixture. Samples were analyzed for nitrogen, in triplicate, by the Kjeldahl method.

Biological value. The biological value (BV) of the unbaked ingredients and of the baked bread was determined by the nitrogen-balance method of Mitchell (1924) and Mitchell and Carman (1926). Twenty weanling rats were placed in separate metabolic cages and fed a stock diet until they reached 80–90 g (av. 88 g). They were then fed the 4.5% egg protein diet for 10 days. During the last seven days of this period, urine and feces were collected daily. Next, the 20 animals were divided into two groups of 10 each, equally distributed as to sex and weight. For 10 days, one group was given the diet of unbaked bread ingredients and the other the baked-bread diet. During the last seven days, urine and feces were collected daily for nitrogen determinations. Samples were digested by the macro-Kjeldahl method and made to volume, and aliquots were taken for micro-Kjeldahl distillation.

Protein efficiency ratio, biological value, true digestibility, net protein value, and net protein utilization of the diets were calculated according to the following formulas:

$$\text{Protein efficiency ratio (PER)} = \frac{\text{Gain in weight, in grams}}{\text{Protein intake, in grams}}$$

(Osborne *et al.*, 1919)

$$\text{Protein value (BV)} = \frac{\text{Food N} - (\text{fecal N} - \text{metabolic N}) - (\text{urinary N} - \text{endogenous N}) \times 100}{\text{Food N} - (\text{fecal N} - \text{metabolic N})}$$

(Mitchell, 1924)

$$\text{True digestibility (TD)} = \frac{\text{Food N} - (\text{fecal N} - \text{metabolic N}) \times 100}{\text{Food N}}$$

(Mitchell, 1924)

$$\text{Net protein value (NPV)} = \frac{\text{Biological value} \times \text{true digestibility}}{100}$$

(Mitchell, 1924)

$$\text{Net protein utilization (NPU)} = \frac{(\text{Body N of test group} - \text{body N of low-protein group} + \text{N consumed by low-protein group}) \times 100}{\text{N consumed by test group}}$$

(Miller and Bender, 1955)

RESULTS AND DISCUSSION

Growth. In all of the experiments (Tables 2, 3, 4), the gain in body weight of animals fed baked bread was 23–53% less than that of the animals fed the unbaked ingredients. By the *t* test, the differences were highly significant in all cases except for the NPU data, in which the difference was significant at the 2% level (average of 8 animals).

Rats fed the steamed bread gained slightly less than did those fed the unbaked ingredients (Tables 2, 3). In two cases, the decreases of 5 and 9% (10- and 28-day periods, respectively) were not significant by the *t* test, while in one case (10-day period) the decrease of 34% was significant at the 2% level.

Protein efficiency ratio. The PER of the baked bread was less than that of the unbaked ingredients (Table 2). The decrease was highly significant whether calculated on a 10-day (40%) or a 28-day period (20%). PER calculated from the NPU data (Table 4) also showed a highly significant decrease of 31%.

The PER of the steamed bread was the same as that of the unbaked ingredients when calculated on a 28-day period, but was 26% less (significant at the 5% level) when calculated on a 10-day period. However, the PER of the steamed bread calculated from NPU data (also a 10-day period) was 6% less than that of the unbaked ingredients, a decrease that was not statistically significant. Apparently, 10 days is too short a period for results to be dependable when changes in gain in weight and in PER are small.

Biological value. The BV (Table 3) of the baked bread was 8.6% less than that of the unbaked ingredients—a highly significant decrease. Because true digestibility of the bread was 3% less than that of the ingredients, the net protein value of the baked bread was decreased by 11%. The BV of the steamed bread was not determined.

Net protein utilization. The NPU of the baked bread, as determined by carcass nitrogen analysis, was 15% less than that of the unbaked ingredients, and that of the steamed bread was 4% less (Table 4).

Effect of baking on protein quality of bread—comparison of methods. By all the criteria used in this study—gain in body weight, protein efficiency ratio, nitrogen balance, and changes in carcass nitrogen—the protein quality of baked whole-wheat bread was significantly lower than that of the unbaked ingredients. Decreases ranged from 9 to 53%, depending on the method used.

The protein quality of steamed bread, however, was the same as, or slightly lower than, that of the ingredients. There was no change in PER (28-day period). Decreases were 4% in NPU and 9% in gain in body weight for a 28-day period. The latter decrease was statistically significant. In two experiments for 10-day periods, decreases of 5 and 34% were obtained in body weight, with corresponding decreases in PER of 6 and 26%. The larger values were statistically significant.

In China, bread is prepared by steaming. For this reason, Adolph and Tsui (1935) compared the growth-promoting qualities of steamed and of baked white wheat bread. They fed the two breads *ad libitum* to weanling rats for 8 weeks; one series received air-dried bread, and a second series received fresh. They concluded that growth was satisfactory and indicated little difference, if any, between the two breads. However, an analysis of the growth curves shows weight gains of 29 and 21 g in 4 weeks, and 54 and 46 g in 8 weeks, for rats fed steamed and baked bread, respectively. Thus the rats fed baked bread gained 28% less in 4 weeks and 15% less in 8 weeks than did those fed steamed bread. This difference in weight gain is in agreement with the present study in which, although the gains in 4 weeks were larger than in the work of Adolph and Tsui—56 g for steamed and 41 g for baked (Table 2)—the rats fed baked bread gained 27% less weight than those fed steamed bread.

Determinations of net protein utilization. In the determination of NPU, Bender and Miller (1953b) suggested a short method for the determination of nitrogen in the carcass, based on the percent of moisture. Assuming that the ratio of nitrogen to water was constant, nitrogen was

Table 2. Protein efficiency ratios of unbaked ingredients and of steamed and baked bread fed to rats.[a]

Diet	Days on experiment	Nitrogen intake (g)	Gain in body weight				Protein efficiency			
			Av. (g)	St. dev.	*t* value	Decrease (%)	Ratio	St. dev.	*t* value	Decrease (%)
Unbaked bread ingredients	10	2.06	16.7	4.4	....	....	1.32	0.25	....	....
	28	6.63	61.0	13.7	....	....	1.56	0.14	....	....
Steamed bread	10	1.92	11.0	4.5	2.88*	34	0.97	0.32	2.73*	26
	28	6.09	55.7	10.5	0.97	9	1.56	0.13	0	0
Baked bread	10	1.73	8.0	2.9	5.28**	52	0.79	0.27	4.50**	40
	28	5.53	40.7	10.7	3.70**	53	1.25	0.16	4.72**	20

[a] Ten rats per group. Diets contained 2.20% nitrogen.

Table 3. Biological value of protein in unbaked ingredients and in baked bread.[a]

	Unbaked ingredients	Baked bread
Initial weight of rats (g)	102	103
Average gain in body weight (g) with standard deviation	19.1±3.6	12.7±4.2
Decrease (%)		34
t value		3.73**
Nitrogen intake (g)	1.670	1.710
Fecal nitrogen (g)	0.165	0.204
Metabolic fecal nitrogen (g)[b]	0.079	0.069
Urinary nitrogen (g)	0.791	0.868
Endogenous urinary nitrogen (g)[b]	0.133	0.135
Biological value and standard deviation[b]	58.5±0.18	53.5±0.19
Decrease (%)		8.6
True digestibility and standard deviation (%)	95.0±2.6	92.2±1.5
t value		2.91*
Net protein value	55.6	49.3

[a] Ten rats per group. Seven-day experimental period.
[b] Determined with same 10 animals over a 7-day period preceding feeding of test diet; 4.5% egg protein diet used.

Table 4. Net protein utilization of unbaked ingredients and of steamed and baked bread.[a]

	Low protein (4.5% egg protein)	Unbaked ingredients	Steamed bread	Baked bread
Gain in body weight per group (g)	27	41	39	29
	29	40	38	33
Decrease (%)[b]		....	5	23
Nitrogen intake per group (g)	2.61	5.63	5.34	5.75
	2.71	5.65	6.01	6.62
Carcass nitrogen per group (g)[c]	6.12	6.37	6.06	5.99
	5.77	5.89	6.02	5.92
Net protein utilization		51	48	43
		50	49	43
Decrease (%)[b]		....	4	15
Protein efficiency ratio	1.67	1.25	1.25	0.87
	1.74	1.22	1.09	0.85
Protein efficiency ratio and standard deviation, average of 8 animals	1.70±0.16	1.24±0.12	1.17±0.18	0.86±0.15
Decrease (%)[b]		....	6	31
t value		....	0.90	5.66

[a] Ten-day experimental period. Two groups of 4 animals each.
[b] Compared with unbaked ingredients.
[c] Determined by nitrogen analysis of carcasses.

calculated from the equation: $y = 2.92 + 0.02x$, where x = age of rat in days and $y = (N \times 100)$ (%) ÷ H_2O (%). Numbers of males and females were equal. From this equation a value of 3.80 is obtained for y for 44-day-old rats. In the present study a value of 3.86 for y was obtained by using 32 rats (equal numbers of males and females), 44 days old, when y was calculated from analyses of the carcasses for nitrogen and moisture. This value is in good agreement with that obtained from the Miller and Bender equation, but a little lower than the 4.09 and 4.16, respectively, from equations by Dreyer (1957) and by Henry and Toothill (1962), for equal numbers of males and females. Forbes and Yohe (1955) reported a ratio of 4.81 for male rats, compared to 4.05 obtained from the equation of Miller and Bender for rats of the same sex and age (about 49 days). Dreyer (1957) stressed the importance of the strain of rat in the use of nitrogen-water ratios.

Dreyer, and Henry and Toothill reported a sex difference in the nitrogen-water ratios. In the present study no significant differences were noted in the ratios, either between sexes or between egg and bread diets. Although male rats had slightly higher carcass nitrogen, carcass moisture, and nitrogen-water ratios than did females on both egg and bread diets, the only value statistically significant by the t test was that for carcass moisture on the bread diets: $t = 2.43$, significant at the 5% level. Both nitrogen and moisture contents of the carcasses for equal numbers of males and females were significantly higher on the bread diets than on the egg diet: $t = 2.90$ and 5.64, respectively.

Variability between duplicates in NPU was greater when carcass nitrogen was calculated from moisture data by using $y = 3.80$, as obtained from the Miller and Bender equation, than when carcass nitrogen was obtained from Kjeldahl data (Table 5). Forbes and Yohe (1955) noted previously that values obtained by the carcass-water method were more variable.

Protein retention as percent of protein ingested was greater when calculated from analysis of excreta than from body analysis.

Table 5. Comparison of net protein utilization calculated from nitrogen determined by Kjeldahl method and from moisture data.

Determination of carcass nitrogen	Net protein utilization		
	Unbaked ingredients	Steamed bread	Baked bread
Kjeldahl	50.8	47.8	43.1
	50.4	49.2	43.2
Av.	50.6	48.5	43.3
From moisture data	55.1	51.5	46.3
	48.7	47.2	38.4
Av.	51.9	49.4	42.4

Net protein values for unbaked bread ingredients and for baked bread were respectively 55.6 and 49.3, compared to 50.5 and 43 for NPU from carcass analysis. This difference has been noted by others (Butterworth, 1962).

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Taste Interrelationships. V. Sucrose, Sodium Chloride, and Citric Acid in Lima Bean Purée[a]

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SUMMARY

A highly trained panel of nine judges determined the taste interactions of suprathreshold concentrations of sucrose, sodium chloride, and citric acid in lima bean purée. The results were in good agreement with previous results with solutions of distilled water, in which: 1) sucrose and citric acid exhibited a mutual masking effect, and 2) sucrose and sodium chloride produced mutual masking except for low levels of sodium chloride (0.05 and 0.10%), which slightly enhanced the apparent sweetness of 0.4 and 0.8% sucrose. Sodium chloride generally depressed sourness, but citric acid significantly increased the saltiness of all levels of sodium chloride. The large amount of individual-judge variation, especially in the acid-salt comparisons, is discussed.

INTRODUCTION

The interaction of sweetness, saltiness, and sourness in solutions of distilled water has been reported by several investigators (Fabian and Blum, 1943; Kamenetzky and Pilgrim, 1958; Beebe-Center *et al.*, 1959; Kamen *et al.*, 1961; and Pangborn, 1960, 1961, 1962). There is no assurance, however, that combinations of pure compounds dispersed in aqueous mediums will elicit similar patterns of response in more complex food systems. For example, fructose is sweeter than sucrose on a weight basis. Although this relationship is valid for water solutions, it was not true when the medium was pear nectar (Pangborn, 1963b). It is much more difficult to ascertain the mechanisms by which primary tastes interact in natural and processed foods because olfactory, kinesthetic, and tactile attributes of the commodity complicate the taste stimuli.

The present investigation was made to establish the taste interrelationships of the following compounds in a natural food product, lima bean purée:

1) Sucrose-citric acid (Studies I & II).
2) Sucrose-sodium chloride (Studies III & IV).
3) Citric acid-sodium chloride (Studies V & VI).

Preparation of samples. Frozen lima beans (USDA Grade A all-green Fordhook variety; Oxnard Frozen Foods Coop., Oxnard, Calif.) were selected as the test material because of availability, uniformity, and freedom from salt or other additives. During the six months required to complete the study, the beans were stored at −15°F in 20-lb lots in polyethylene bags in sealed cardboard containers. Purées were prepared daily between 1 and 2 P.M. by adding 600 g of frozen beans to 500 ml of boiling distilled water and cooking for 20 min. The cooked beans were drained for 5 min, combined with 500 ml of distilled water, and puréed in an electric blender for 100 sec at 110 volts. Weighed quantities of sucrose, sodium chloride, or citric acid were added to weighed portions of the purée, which was placed in covered glass beakers in a water bath maintained at 174±2°F during the time of serving (2:30 to 3:00 P.M.). The concentrations of the additives, which had been pretested, ranged from threshold to very intense levels for lima beans. Samples were served in coded, black-lined, heavy porcelain cups that were placed in polystyrene blocks to maintain the temperature between 165 and 170°F during the tasting period.

Taste panel. Six men and seven women judges were used. All had extensive experience in tasting aqueous solutions and

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one year of experience in tasting lima bean purée. The original selection was made on the basis of a judge's consistent ability to recognize small differences in sweetness, sourness, and saltiness in aqueous solutions (Pangborn, 1962). Judges were seated in individual, partitioned booths maintained at 70±2°F. Each judge received four pairs of purée in randomized order at each test session except in Study VI, where five pairs were presented. All testing was done under low, red illumination to eliminate visual clues, since the purées containing citric acid were a lighter green than those containing sucrose or salt. Distilled water was provided for oral rinsing, but judges were instructed not to swallow any water or purée. Testing sessions were held Monday through Friday, and panel members were informed of their progress after each session. Individuals who were unable to attend a testing period were required to have a "make-up" the following day so that, within a study, all samples were tested by all subjects an equal number of times. As indicated in Table 5, not all judges were available for all studies.

A method of paired-comparison constant-stimulus, described previously (Pangborn, 1961), was used. Within a pair, both samples contained the secondary taste compound whereas only one contained the primary compound. Judges were required to circle the number of the sample within each pair that contained the greater intensity of the secondary taste sensation. In addition, judges indicated the degree of difference in taste intensity within each pair on a four-point scale of "slight, moderate, large, or extreme." These descriptive terms were converted to integers of −7, −5, −3, −1, +1, +3, +5, and +7, and analysis of variance was applied to the data. Chi-square analysis was applied to determine significance of difference in the paired selections.

RESULTS

Study I. Effect of sucrose on apparent sourness of citric acid. The frequency of selection of the sample with the greater sourness within each pair is given in Table 1. As the amount of sucrose was increased, a greater number of selections indicated that the sweetened sample was less sour. This reduction in sourness by sucrose occurred to the same degree in all four levels of citric acid. The average difference scores for this relationship (Fig. 1) were all negative, verifying that increasing amounts of sucrose decreased the apparent sourness of all levels of citric acid to a similar degree. Analysis of variance of the difference scores indicated that the judges contributed substantially to the amount of variation in the data and reacted differentially to both the citric acid and the sucrose (Table 2). No variation was attributable to replications, indicative of the reproducibility of the responses. Table 5 shows that although all judges ascribed negative average total difference scores, the intensity of difference ranged from −0.31 to −2.16.

Study II. Effect of citric acid on apparent sweetness of sucrose. Selections of samples with greater sweetness within pairs showed that levels of 0.1 and 0.2% citric acid significantly reduced apparent sweetness (Table 1). Higher levels of sucrose were reduced slightly more than were lower levels (Fig. 2). The analysis of variance

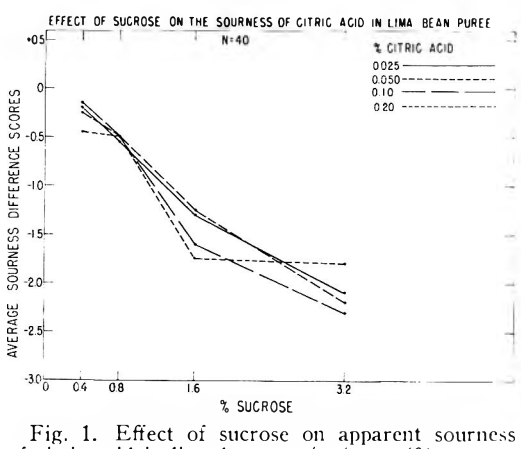

Fig. 1. Effect of sucrose on apparent sourness of citric acid in lime bean purée (n = 40).

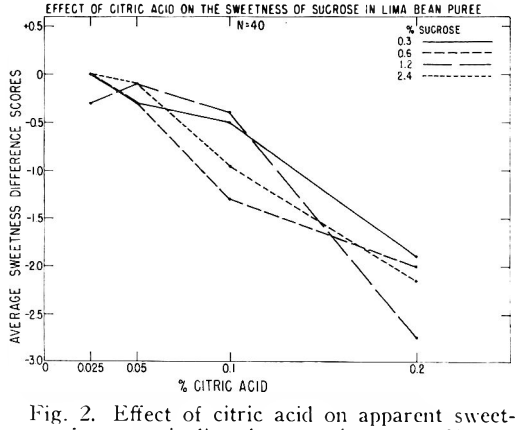

Fig. 2. Effect of citric acid on apparent sweetness of sucrose in lima bean purée (n = 40).

Table 1. Taste intensity of sucrose, sodium chloride, and citric acid in lima bean purée. Frequency of selection of sample without primary as most intense within each pair.

Primary: Sucrose (%)	I. Sucrose sourness (n=40) 0.4	0.8	1.6	3.2	Total
% Citric acid					
.025	24	27[a]	36[c]	36[c]	123[c]
.05	24	25	33[c]	35[c]	117[c]
.10	22	29[b]	37[c]	40[c]	128[c]
.20	25	28[a]	35[c]	33[c]	121[c]
Total	95[a]	109[c]	141[c]	144[c]	489[c]

Primary: Citric acid (%)	II. Citric sweetness (n=40) .025	.05	.10	.20	Total
% Sucrose					
0.3	20	21	25	34[c]	100[b]
0.6	20	24	36[c]	35[c]	115[c]
1.2	23	21	26	38[c]	108[c]
2.4	25	24	35[c]	37[c]	121[c]
Total	88	90	122[c]	144[c]	444[c]

Primary: Sucrose (%)	III. Sucrose saltiness (n=36) 0.2	0.4	0.8	1.6	Total
% NaCl					
.05	18	17	27[b]	31[c]	93[c]
.10	22	23	25[a]	32[c]	102[c]
.20	20	18	32[c]	32[c]	102[c]
Total	60	58	84[c]	95[c]	297[c]

Primary: NaCl (%)	IV. Sodium chloride sweetness (n=36) .05	.10	.20	.40	Total
% Sucrose					
0.4	13	10[a]	16	16	55[b]
0.8	17	15	18	20	70
1.6	14	14	20	24	72
Total	44[a]	39[b]	54	60	197

Primary: Citric acid (%)	V. Citric saltiness (n=45) .025	.05	.10	.20	.40	Total
% NaCl						
.03	21	29	21	25	23	119
.06	24	23	20	24	23	114
.12	27	27	18	21	21	114
.25	31[a]	17	22	25	24	119
Total	103	96	81	95	91	466[a]

Primary: NaCl (%)	VI. Sodium chloride sourness (n=36) .05	.10	.20	.40	Total
% Citric acid					
.035	29[c]	26[a]	22	24	101[c]
.070	28[b]	26[a]	24	23	101[c]
.140	26[a]	24	27[b]	21	98[c]
.280	25[a]	22	29[c]	21	97[c]
Total	108[c]	98[c]	102[c]	89[b]	397[c]

[a, b, c] Respectively significant differences within pairs at P = 0.05, 0.01, and 0.001.

Table 2. Analysis of variance of difference scores. Interaction of sucrose and citric acid.

Source of variation	Degrees of freedom	I. Sucrose—sourness[a]	II. Citric—sweetness[b]
		Mean square	Mean square
(S) Sucrose	3	116.62***	1.72
(C) Citric	3	0.42	151.72***
(J) Judges	9	30.59***	25.80***
(R) Replications	3	0.72	4.34*
S × C	9	1.49	4.16**
S × J	27	9.44***	1.78
S × R	9	1.01	1.02
C × J	27	5.30***	7.77***
C × R	9	2.17	1.31
J × R	27	2.39	1.70
S × C × R	27	1.27	1.18
S × C × J	81	1.70	3.23***
S × R × J	81	1.50	1.13
C × R × J	81	1.79	1.40
Remainder	243	1.58	1.45

[a] Effect of sucrose on sourness of citric acid.
[b] Effect of citric acid on sweetness of sucrose.
*, **, *** Respectively sig. at P = 0.05, 0.01, 0.001.

demonstrated a significant interaction between the two compounds (Table 2). Judges contributed significantly to the observed variation in the data and reacted differently to the acid but not to the sugar. The slight degree of significance due to replication suggests that some judges were inconsistent in their responses upon repeated tasting, or that some other factor influenced the scoring. Only one judge (Judge 9) indicated a general enhancement of sweetness by the acid (Table 5). Judges 1, 2, 3, 10, and 11 averaged high negative values, and greatly influenced the average panel response.

Study III. Effect of sucrose on apparent saltiness of sodium chloride. The higher levels of sucrose (0.8 and 1.6%) decreased the saltiness of 0.05, 0.10, and 0.20% salt (Table 1). As illustrated in Fig. 3, the lowest salt level was depressed the least. There was greater agreement in opinion between judges in this study than in any other, as evidenced by lower judge variation (Table 3) and a smaller range in the average total difference scores (Table 5).

Study IV. Effect of sodium chloride on apparent sweetness of sucrose. Lower levels of sodium chloride enhanced and higher levels depressed apparent sweetness (Table 1, Fig. 4). The apparent sweetness of the highest concentration of sucrose, 1.6%, was reduced substantially by additions of 0.2 and 0.4% sodium chloride. Although the same judges participated in both studies, the analysis of variance showed that variability attributable to judges was greater in Study IV than in Study III (Table 3). In addition, all interactions involving judges were significant. Table

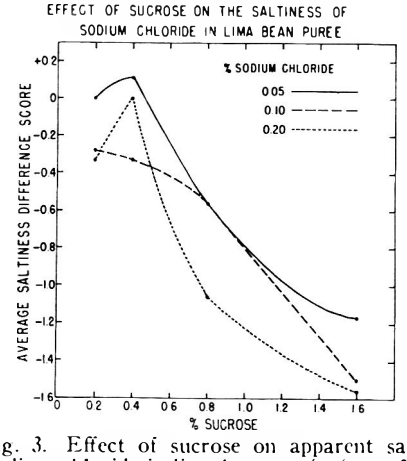

Fig. 3. Effect of sucrose on apparent saltiness of sodium chloride in lima bean purée (n = 36).

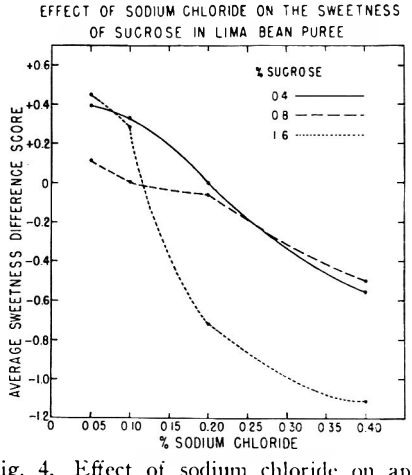

Fig. 4. Effect of sodium chloride on apparent sweetness of sucrose in lima bean purée (n = 36).

Table 3. Analysis of variance of difference scores. Interaction of sucrose and sodium chloride.

Source of variation	III. Sucrose—saltiness [a]		IV. Sodium chloride—sweetness [b]	
	Degrees of freedom	Mean square	Degrees of freedom	Mean square
(S) Sucrose	3	39.62***	2	3.68
(N) NaCl	2	4.45*	3	24.33***
(J) Judges	8	7.48***	8	78.23***
(R) Replications	3	0.21	3	0.63
S × N	6	1.07	6	2.85*
S × J	24	4.48***	16	3.40**
S × R	9	1.47	6	1.15
N × J	16	1.14	24	8.30***
N × R	6	0.55	9	1.01
J × R	24	1.49	24	2.20*
S × N × R	18	0.97	18	0.98
S × N × J	48	1.50*	48	2.72***
S × R × J	72	1.61**	48	0.88
N × R × J	48	1.02	72	1.30
Remainder	144	1.01	144	1.27

[a] Effect of sucrose on saltiness of sodium chloride.
[b] Effect of sodium chloride on sweetness of sucrose.
*, **, *** Respectively sig. at P= 0.05, 0.01, and 0.001.

5 shows the range in average difference scores categorized by individual judges. Judges 2, 3, and 13 assigned high negative values, whereas the remaining six judges assigned positive difference scores.

Study V. Effect of citric acid on apparent saltiness of sodium chloride. In this study, judges were sharply divided in their reaction to apparent saltiness. As indicated in Table 5, five of the nine subjects found saltiness to be decreased by increasing amounts of citric acid, whereas four subjects reported marked enhancement. The magnitude of this subdivision can be seen in the following table:

% citric acid	0.025	0.05	0.1	0.2	0.4
			average difference scores		
Group A. n = 100 (5 subjects)	−0.16	−0.18	−0.52	−1.60	−1.62
Group B. n = 80 (4 subjects)	−0.05	0	+1.05	+2.58	+4.45

Although the majority of the panel reported depression of saltiness, the scores of the minority carried more weight, so that the average effect was enhancement (Fig. 5). Statistical analysis of these data showed the great extent to which judge variation influenced the results (Table 4). The insignificant variance for replications attests to the ability of the panel to replicate the responses; however, J × R was significant, as was the interaction of C × N × J. This study is currently being repeated with water solutions and green bean purée, using the same experimental subjects.

Study VI. Effect of sodium chloride on apparent sourness of citric acid. In general, sodium chloride decreased apparent sourness (Fig. 6). The lower levels of citric acid produced a pattern quite dissimilar to the patterns for higher levels. Analysis of variance suggested that the large judge-effects may have been responsible for the response pattern. Panel replications and the interaction of J × R were not significant, indicating a reproducibility of individual judgment, even though the interaction of judges × sodium chloride was significant.

DISCUSSION

Responses to combinations of sucrose, sodium chloride, and citric acid in lima bean purée were in complete agreement with those obtained previously in solutions of distilled water (Pangborn, 1960, 1961, 1962). Although the range of concentrations tested was greater in water than in purée, the patterns of response were similar, if not identical.

The most clear-cut results were those involving interactions of sweetness and sourness, where mutual masking occurred in water (Pangborn, 1960, 1961, 1963a), in pear nectar (Pangborn, 1960), in canned tomato juice (Pangborn and Chrisp, 1964),

Table 4. Analysis of variance of difference scores. Interaction of citric acid and sodium chloride.

Source of variation	V. Citric—saltiness[a]		VI. Sodium chloride—sourness[b]	
	Degrees of freedom	Mean square	Degrees of freedom	Mean square
(C) Citric	4	42.43***	3	2.12
(N) NaCl	3	2.93	3	4.99
(J) Judges	8	192.21***	8	82.42***
(R) Replications	4	0.96	3	1.64
C × N	12	2.58*	9	1.83
C × J	32	50.29***	24	3.18
C × R	16	0.80	9	3.04
N × J	24	2.93**	24	10.72***
N × R	12	2.12	9	2.36
J × R	32	3.21***	24	1.32
C × N × R	48	0.89	27	1.80
C × N × J	96	2.25**	72	3.79**
C × R × J	128	1.61	72	1.27
N × R × J	96	1.33	72	1.58
Remainder	384	1.34	216	2.25

[a] Effect of citric acid on saltiness of sodium chloride.
[b] Effect of sodium chloride on sourness of citric acid.
*, **, *** Respectively sig. at P= 0.05, 0.01, and 0.001.

as well as in lima bean purée. In variance with the results obtained in our laboratories are those of Fabian and Blum (1943), who used a matching technique, and Kamen *et al.* (1961), who presented samples singly. Although those investigators agreed that sucrose reduced the intensity of sourness in water solutions, they observed that citric acid generally increased the sweetness of sucrose. In Fabian and Blum's study, various acids affected the sweetness of glucose, sucrose, and fructose differentially, but all sugars decreased the sourness of all acids. Pangborn (1963a) retested much of those data, using a paired-comparison constant-stimulus procedure, and observed that citric, tartaric, lactic, and acetic acids significantly reduced the sweetness of glucose, sucrose, lactose, and fructose to approximately the same extent.

Agreement on the general interaction-effects of sweetness and saltiness was excellent among various carriers, but not between investigators. In our laboratory, in

Table 5. Average difference scores assigned by individual judges.[a]

Judge	Sex	I Sucrose—sourness	II Citric—sweetness	III Sucrose—saltiness	IV NaCl—sweetness	V Citric—saltiness	VI NaCl—sourness
		n = 64	n = 64	n = 48	n = 48	n = 100	n = 64
1	F	−1.56	−1.44	−0.79	1.25	−1.06	−2.75
2	F	−1.25	−1.13	−0.58	−1.38	0.88	0.34
3	F	−1.63	−1.75	−1.29	−1.71	−1.42	−1.50
4	F	−0.56	−0.75	.....	.....	−0.48	−0.31
5	F	−0.78	−0.34	.....	.....	2.08	0.03
6	F	−0.09	−0.22	−0.71	0.46	.....	.....
7	F	.....	.....	−0.13	0.46	.....	.....
8	M	−0.72	−0.19	−0.04	0.83	−0.88	−1.66
9	M	−0.31	0.13	−0.54	0.71	−0.24	0.63
10	M	−2.16	−1.41	.....	.....	2.32	−1.72
11	M	−1.81	−1.06	.....	.....	1.14	−1.34
12	M	.....	.....	−0.96	0.54	.....	.....
13	M	.....	.....	−0.38	−2.21	.....	.....
Δ		2.07	1.88	1.25	3.46	3.74	3.38

[a] Negative scores indicate the compound depressed apparent taste intensity. Positive scores indicate the compound enhanced apparent taste intensity.

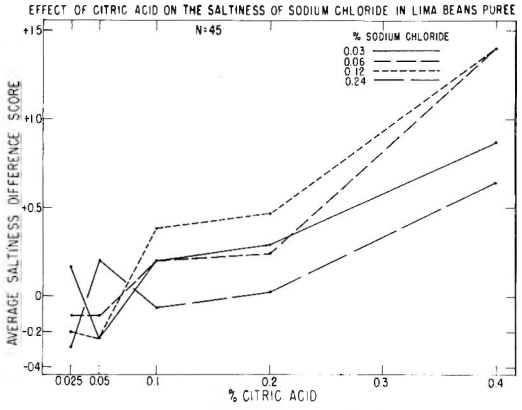

Fig. 5. Effect of citric acid on apparent saltiness of sodium chloride in lime bean purée (n = 45).

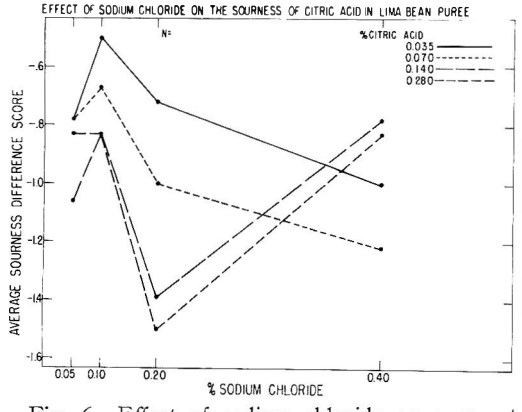

Fig. 6. Effect of sodium chloride on apparent sourness of citric acid in lima bean purée (n = 36).

water solutions, canned tomato juice, and lima bean purée, sucrose and sodium chloride exhibited mutual masking effects, except for a slight enhancement of low levels of sucrose by low levels of sodium chloride. For solutions of distilled water, Fabian and Blum (1943) reported that sodium chloride increased the sweetness of all sugars but all sugars decreased saltiness. Beebe-Center *et al.* (1959) observed mutual masking except for slight enhancement of solutions of 1% sucrose by up to 3% sodium chloride. Kamen *et al.* (1961) concluded that the reduction of the sweetness of sucrose by sodium chloride occurred primarily in solutions that were high in both salt and sugar. For the lower sugar concentrations, the various levels of salt had relatively little effect. Those investigators further reported an absence of an overall enhancing or masking effect in their study of sucrose on saltiness.

Citric acid increased the saltiness of sodium chloride in water solutions (Fabian and Blum, 1943; Kamen *et al.*, 1961), in canned tomato juice (Pangborn and Chrisp, 1964), and in lima bean purée. Sodium chloride, on the other hand, generally decreased sourness in all mediums. Kamen *et al.* (1961) noted that the highest levels of salt tended to enhance the sourness of the lower concentrations of citric acid but reduced the sourness of the higher acid concentrations. They were unable to give an explanation for this non-monotonic function but observed that the results of the effect of salt on sourness were more complex than those from other interactions.

As early as 1899 Heymans reported that sodium chloride raised the threshold for hydrochloric acid, and vice versa. Only one subject was employed in his investigation. Subsequently, Cragg (1937), using an undisclosed number of subjects, reported that the addition of 0.2*M* sodium chloride had no effect on the sourness of hydrochloric acid. According to Hahn and Ulbrich (1948), thresholds for hydrochloric or acetic acids were unaffected by the addition of threshold levels of sodium chloride. Thresholds for sodium chloride, however, were decreased in one out of three subjects for hydrochloric acid and in two out of four subjects for acetic acid. Anderson (1950) observed that tartaric acid raised the threshold for sodium chloride in both experimental subjects, but the acid threshold increased in the presence of salt for only one of the subjects.

It is apparent that a lack of agreement between subjects could be the major contributing factor in producing divergent results between laboratories. To our knowledge, none of the investigators cited herein has separated their data for analysis of individual judge effects. In some studies, only one or two judges were used. Fabian and Blum (1943) used results from ten subjects but did not analyze for judge or replication effects. Kamen *et al.* (1961) drew his 960 judgments from 700 participants, so that few people tasted more than

once. Nevertheless they stated there was a significant between-judge variation in their sodium chloride-sourness study. Using the same single-stimulus method as did Kamen *et al.*, Pangborn (1962) observed highly significant variation attributable to treatments, judges, replications, and all interactions. However, when the same judges used the paired-stimulus technique, significant F ratios were obtained for treatments and judges but not for replications (d.f. = 9) or for any interactions involving replications.

Thus it can be concluded that the factors accounting for contradictory responses to taste mixtures between laboratories include: 1) individual variability in response to taste mixtures; 2) lack of reproducibility of judgment upon repeated testing; 3) range of concentrations tested; and 4) the psychophysical tests used.

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