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CONTENTS

Utilization of Some Carbon and Nitrogen Sources by *Pseudomonas fluorescens*[a]

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SUMMARY

All test cultures of *Pseudomonas fluorescens* established growth in a basal medium (0.02% Mg SO_4, 0.1% KH_2PO_4, and 0.5% glucose) with $NH_4H_2PO_4$, $NaNH_4HPO_4$, or $(NH_4)_2HPO_4$ as the sole source of nitrogen. NH_4Cl, NH_4NO_3, and $(NH_4)_2SO_4$ supported growth of the majority of the cultures. Complex organic N substrates were readily utilized by all test cultures. Glutamic acid, leucine, and proline were satisfactory sources of both nitrogen and carbon for all test cultures. With $NH_4H_2PO_4$ as the sole source of nitrogen, fructose, galactose, glucose, mannose, maltose, trehalose, starch, arabitol, erythritol, glycerol, inositol, *scyllo* inosose, citric acid, malic, pyruvic, caprylic or capric acid supported growth of all cultures. 1-Nonanol, 1-decanol, and 1-hendecanol were satisfactory carbon sources.

In recent years numerous studies have been conducted on the growth characteristics and biochemical activities of bacteria that are capable of proliferating in foods at refrigerated temperatures. In many instances these bacteria are responsible for flavor and odor defects of foods stored under refrigeration for long periods. Even without production of undesirable flavors and odors they may become a problem when viable counts above the legal maximum develop. Many of the psychrophilic bacteria isolated from foods belong to the genus *Pseudomonas*. The predominant species in this genus are *P. fluorescens*, *P. fragi*, and *P. putrefaciens*. These bacteria may cause a variety of flavor and odor defects in foods by their proteolytic and or lipolytic activities. Although many investigations have been carried out on different aspects of these pseudomonads, few have been conducted on their nutritional needs. Den Dooren de Jong (1926) reported on the growth response of a group of common bacteria, including one strain of *P. fluorescens*, to various sources of carbon and nitrogen in a synthetic medium. Reinbold *et al.* (1953a,b) determined the carbon and nitrogen requirements of *P. putrefaciens*. Hussong *et al.* (1937), Ousley (1957), and Pereira and Morgan (1957) reported on the nutritional requirements of *P. fragi*. This study was initiated to determine the growth response of various strains of *P. fluorescens* to various sources of carbon and nitrogen. These data then were used to compare the utilization of various carbon and nitrogen sources by various strains of *P. fluorescens*, *P. fragi*, and *P. putrefaciens*.

EXPERIMENTAL METHODS

Cultures. Seven strains of *P. fluorescens* of the stock culture collection of the Dairy Science Department of the A. and M. College of Texas were used. The identity of the cultures conformed to the description in Bergey's Manual (Breed *et al.*, 1957). The stock cultures were carried in litmus milk and were transferred weekly. The inoculum for the experimental media was prepared from cells grown 24 hr at 25°C on slants of plate-count agar. The cells were removed from the slants and washed three times in sterile distilled water. Following centrifugation the final cell suspension was diluted with sterile distilled water to deliver approximately 10^5 organisms (1 drop from a standard 0.1-ml pipette) per 10 ml of test medium. The dilution factor was established by correlating the number of viable cells by plating on plate-count agar with the density as measured with a Spec-

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tronic 20 colorimeter. Incubation of the test media was 5 days at 25°C. On completion of the first 5-day incubation period, one drop of the test culture was transferred to a tube of the same, but sterile, medium, which was then incubated 5 days at 25°C. In this manner two successive transfers were made at 5-day intervals. All transfers were done in duplicate. Following each incubation period, growth was measured photometrically with a Spectronic 20 at 440 mμ. The extent of growth of the duplicates compared closely and the average of the two values (% transmittance) was used as the reading for that transfer. The % transmittance reported is the average of the three readings.

Test media. The effect of various N sources on the growth response of *P. fluorescens* was determined in a glucose-salt medium consisting of 0.02% $MgSO_4$, 0.1% KH_2PO_4, and 0.5% glucose. In the study on the effect of C sources on the growth of *P. fluorescens* the basal medium contained 0.1% $NH_4H_2PO_4$, 0.02% $MgSO_4$, and 0.1% KH_2PO_4. All test media were adjusted to pH 7.0, sterilized by filtration through Seitz filters, and dispensed in 10-ml quantities in sterile test tubes. The growth response of the cultures to fatty acids and water-insoluble alcohols was measured on solid media of the same composition as the liquid basal medium ($NH_4H_2PO_4$, $MgSO_4$, and KH_2PO_4) except for the addition of 2.5 g of $CaCO_3$ and 20 g of agar per L. The sterile (Seitz-filtered) volatile acids were added just before the plates were poured. The other fatty acids were added to the basal medium before autoclaving 15 min at 121°C.

The plates were inoculated with one drop of inoculum. Incubation of plates was for 5 days at 25°C. Any growth on the plate was streaked on a second plate of the same medium and incubated 5 days at 25°C. This procedure was repeated once. Seventy-two compounds including pentoses, mono-, di-, tri-, and polysaccharides, polyols, organic acids, and alcohols were tested as source of C for *P. fluorescens*. Six inorganic N sources, 18 amino acids, various proteins, and other complex organic compounds were tested as N sources.

RESULTS

Utilization of N compounds. The 6 inorganic N sources tested supported the growth of a majority of the strains of *P. fluorescens* (Table 1). $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$ served as suitable N sources for all test cultures. The growth responses of the individual strains to $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$ were very similar although the medium with $(NH_4)_2HPO_4$ contained considerably more N (2.12 vs 1.21 mg/10 ml) than that with $NH_4H_2PO_4$. A similar situation was observed in comparing the growth responses of strains 2, 3, 11, 70, E, and L11 to $NaNH_4HPO_4$, NH_4Cl, and $(NH_4)_2SO_4$. The media with NH_4Cl and $(NH_4)_2SO_4$ contained more than twice as much N as that with $NaNH_4HPO_4$. The various strains varied considerably in growth response to the individual N sources.

As shown in Table 2, all complex N sources supported growth of all test cultures of *P. fluorescens*. Albumin and gelatin, however, were less effective in supporting growth than the other N sources. In contrast to the results obtained with the inorganic N sources, the differences in growth response of the various cultures to the individual complex N sources was much more limited.

Tables 3 and 4 show the growth responses of 7 strains of *P. fluorescens* to 18 single amino acids added to a basal medium with 0.5% glucose. In one study (Table 3) the amount of each amino acid added represents the calculated level present in the casein of 10 ml of milk (Neurath and Bailey, 1954); in the other (Table 4) each amino acid was added to give an N concentration of 0.1 mg per 10 ml of medium. With few exceptions, all cultures were capable of establishing growth in the basal medium with one of the 18 amino acids added. In general, the different cultures showed little variation in growth response to the individual amino acids. The poor growth response of the cultures to cystine, methionine, and tryptophan in one series of experiments (Table 3) was due at least in part to a low concentration of available N. The extent of growth was usually greater at the higher levels of added N. The data in Table 4 indicate that growth response to cystine, methionine, and tryptophan was better at higher concentrations of these amino acids. On the other hand, with the same amount of added N, certain amino acids (aspartic acid, glycine, proline) supported growth much better than others (cystine, methionine, tryptophan). The levels of growth with the same substrate did not vary greatly between strains.

Each of the 18 amino acids (0.1 mg N per 10 ml medium) was also tested as the sole source of both C and N for *P. fluorescens*. A majority of the cultures showed a fair to good growth response when glutamic acid, leucine, or proline was used as the sole source of both C and N. Cystine, glycine, methionine, and tryptophan failed to support growth of any of the test cultures. The growth response to the other amino acids was poor or negative.

Utilization of carbon compounds. Seven of 35 carbohydrates tested, including fructose, galactose, glucose, mannose, trehalose, starch, and glycerol, supported good growth of all cultures of *P. fluorescens*. Cellobiose, lactose, melibiose, turanose, melezitoze, xylan, and dulcitol failed to support growth

Table 1. Utilization of inorganic nitrogen sources by 7 strains of *Pseudomonas fluorescens*.

Nitrogen [a] source	N in 10 ml medium (mg)	Strain						
		2	3	11	53	70	E	L11
$NH_4H_2PO_4$	1.21	43[b]	21	20	33	19	41	33
$NaNH_4HPO_4$	1.02	98	32	30	61	32	43	36
$(NH_4)_2HPO_4$	2.12	45	19	27	35	21	40	25
NH_4Cl	2.62	98	25	35	100	30	53	39
NH_4NO_3	1.75	100	59	72	61	26	100	43
$(NH_4)_2SO_4$	2.12	100	23	38	100	33	52	34

[a] Nitrogen source (0.1%) added to glucose-salt medium.
[b] % light transmittance, uninoculated blank = 100.

Table 2. Effect of complex organic nitrogen sources on the growth of 7 strains of *Pseudomonas fluorescens*.

Organic N [a] source	N in 10 ml medium (mg)	Strain						
		2	3	11	53	70	E	L11
Albumin	1.57	95[b]	87	90	84	86	77	91
Casein	1.39	31	52	53	31	32	21	44
Casamino acids	1.0	34	25	35	39	39	29	30
Beef extract	0.83	26	41	48	37	53	47	46
Gelatin	1.81	82	86	86	90	89	89	85
Peptone	1.62	59	45	43	51	43	54	41
Proteose peptone	1.44	53	46	38	47	41	46	41
Tryptone	1.31	49	32	29	44	27	35	28
Yeast extract	0.87	43	32	27	38	26	34	26

[a] Nitrogen source (0.1%) added to glucose-salt medium.
[b] % light transmittance, uninoculated blank = 100.

Table 3. Effect of 18 amino acids supplemented with glucose on the growth of 7 strains of *Pseudomonas fluorescens*.

Amino acid added [a]	N in 10 ml medium (mg)	Strain						
		2	3	11	53	70	E	L11
L-Alanine	0.050	88[b]	86	83	86	81	88	83
L-Arginine	0.130	87	70	71	72	69	75	70
L-Aspartic acid	0.075	61	75	71	69	69	76	71
L-Cystine	0.004	95	96	97	97	95	100	100
L-Glutamic acid	0.213	77	78	78	79	75	81	80
Glycine	0.037	74	78	77	76	78	82	80
L-Histidine	0.084	70	74	79	77	77	81	79
L-Isoleucine	0.065	86	84	82	86	57	81	83
L-Leucine	0.098	80	91	82	84	82	86	83
L-Lysine	0.157	78	77	78	83	81	79	77
L-Methionine	0.026	92	94	88	92	84	93	92
L-Phenylalanine	0.043	85	89	87	89	85	89	86
L-Proline	0.129	64	68	67	65	67	70	67
L-Serine	0.084	79	83	79	81	77	84	81
L-Threonine	0.058	89	92	86	89	84	87	84
L-Tryptophan	0.023	96	95	94	100	91	97	96
L-Tyrosine	0.049	87	90	86	87	83	90	86
L-Valine	0.086	88	84	81	83	80	85	81
All	1.411	43	37	38	37	28	43	27

[a] Compound added to glucose-salt medium.
[b] % light transmittance, uninoculated blank = 100.

of any of the test cultures. The data in Table 5 can be summarized as follows: a) 5 of the 7 strains of *P. fluorescens* showed little or no growth response to a majority of the pentoses tested, though strains 3 and E were capable of utilizing most of the pentoses; b) except for sorbose, most monosaccharides supported good growth of all test strains; c) except for trehalose and starch, the di-, tri-, and polysaccharides tested failed to support the growth of most test strains; and d) a majority of the strains of *P. fluorescens* (except strains 2 and 53) were capable of utilizing most of the polyols as the sole source of C. Of the 22 organic acids tested, ten (oxalic, formic, acetic, propionic, butyric, valeric, lauric, palmitic, stearic, and oleic acid) failed to support the growth of any of the test cultures. Most of the other organic acids (Table 6) supported good growth of strains 3, 11, 70, E, and L11. Five of the 15 alcohols (methanol, 2-propanol, 2 methyl-2-butanol, 1-heptanol, and 2-octanol) were not utilized as sole source of C. Nonanol, decanol, and hendecanol, however, supported the growth of all test strains (Table 7). The other alcohols were not satisfactory C sources for most strains of *P. fluorescens*. Strain E was capable of attaining good growth with each one of nine alcohols as sole source of C.

DISCUSSION AND CONCLUSIONS

In general, the results show that inorganic N sources, complex organic substrates, and each of 18 amino acids were suitable N sources for a majority of the test cultures of *P. fluorescens*. Extent of growth with $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$ was comparable to that observed with some of the complex organic N substrates. The data in Table 1 indicate that not all strains grew when NH_4Cl, NH_4NO_3, and $(NH_4)_2SO_4$ were used as the sole source of N, yet they all grew when the phosphate salts were used. Pereira and Morgan (1957) observed a similar phenomenon with some strains of *P. fragi* when certain ammonium salts were used as the sole source of N. Although little is known about the effect of anions and cations and ion imbalance on the individual strains of a species, the possibility exists that the anions may be responsible for this observation. The growth response of *P. fluorescens* to various N sources was in many instances similar to that reported for *P. fragi* (Ousley, 1957; Pereira and Morgan, 1957), but differed greatly from that of *P. putrefaciens* (Reinbold *et al.*, 1953a). Inorganic ammonium salts and most amino acids except for aspartic acid, glutamic acid, leucine, isoleucine, and tyro-

Table 4. Effect of 18 amino acids supplemented with glucose on the growth of 7 strains of *Pseudomonas fluorescens*.

Amino acid added [a]	Strain						
	2	3	11	53	70	E	L11
L-Alanine	79 [b]	75	77	77	79	78	73
L-Arginine	85	75	72	76	70	75	73
L-Aspartic acid	60	69	70	64	65	69	70
L-Cystine	89	90	88	86	89	90	89
L-Glutamic acid	79	79	77	80	78	84	84
Glycine	64	65	69	67	67	69	69
L-Histidine	75	77	80	79	79	82	80
L-Isoleucine	81	80	79	79	69	72	75
L-Leucine	79	87	80	83	81	82	82
L-Lysine	78	77	77	79	79	73	75
L-Methionine	87	88	85	87	80	89	87
L-Phenylalanine	83	81	80	82	81	80	79
L-Proline	69	70	67	66	68	75	75
L-Serine	75	77	77	80	79	75	75
L-Threonine	75	76	75	75	75	77	79
L-Tryptophan	89	87	85	83	85	85	87
L-Tyrosine	85	81	80	91	80	81	83
L-Valine	81	79	77	77	81	84	77

[a] Amino acids (0.1 mg N/10 ml medium) added to glucose-salt medium.
[b] % light transmittance, uninoculated blank = 100.

Table 5. Effect of 28 carbohydrates on the growth of 7 strains of *Pseudomonas fluorescens*.

Carbohydrate added [a]	Strain						
	2	3	11	53	70	E	L11
D-Arabinose	100 [b]	98	100	70	100	86	100
L-Arabinose	58	56	100	67	97	61	100
D-Lyxose	100	79	100	97	100	93	100
D-Ribose	100	67	100	100	100	65	100
D-Xylose	100	60	67	97	56	60	61
L-Fucose	100	67	100	73	100	75	100
L-Rhamnose	100	63	100	100	100	73	100
D-Fructose	75	56	64	73	71	70	68
D-Galactose	70	47	75	83	61	60	74
D-Glucose	46	51	45	78	31	46	40
D-Mannose	78	51	61	85	56	71	58
L-Sorbose	100	98	100	98	97	100	71
Maltose	94	93	94	90	93	90	94
Sucrose	100	93	100	100	100	71	100
Trehalose	85	57	69	77	59	69	49
Raffinose	89	100	100	100	100	100	97
Dextrin	100	100	100	100	100	100	97
Inulin	93	97	100	100	100	100	96
Starch	86	84	82	87	80	75	80
Salicin	100	88	100	100	100	100	100
Adonitol	100	61	65	96	58	58	63
L-Arabitol	97	62	71	96	70	65	65
Erythritol	97	59	63	96	57	64	58
Glycerol	78	49	58	81	55	51	48
D-Mannitol	100	63	64	100	67	64	59
D-Sorbitol	100	60	100	100	100	60	100
Inositol	94	60	71	66	73	62	70
Scyllo-inosose	97	71	83	79	80	73	81

[a] Compound (0.1%) added to $NH_4H_2PO_4$-salt medium.
[b] % light transmittance, uninoculated blank = 100.

Table 6. Effect of 12 organic acids on the growth of 7 strains of *Pseudomonas fluorescens*.

Acid added [a]	Strain						
	2	3	11	53	70	E	L11
Cis-aconitic	100 [b]	97	78	100	49	85	73
Citric	67	82	63	76	52	61	53
Fumaric	100	71	68	100	56	65	68
Isocitric	100	97	86	93	88	90	86
α-Ketoglutaric	100	52	49	81	41	57	52
Lactic	100	61	59	100	44	42	54
Malic	40	49	47	58	35	42	36
Pyruvic	97	91	71	98	48	59	62
Succinic	100	71	66	100	42	63	62
Compounds added to solid media							
Caproic	0 [c]	2.3	0	0	1.7	0	1.7
Caprylic	2.3	3.3	2.7	2.7	3.0	2.0	2.0
Capric	3.0	3.3	3.3	2.7	3.3	3.0	3.0

[a] Compound (0.1%) added to $NH_4H_2PO_4$-salt medium.
[b] % light transmittance, uninoculated blank = 100.
[c] Average of 3 readings (0 = no growth, 1 = slight growth, 2 = medium growth, 3 = good growth, 4 = abundant growth).

Table 7. Effect of 10 alcohols on the growth of 7 strains of *Pseudomonas fluorescens*.

Alcohol added [a]	Strain 2	3	11	53	70	E	L11
Ethanol	100 [b]	72	100	100	100	76	70
1-Propanol	100	68	100	100	100	69	81
1-Butanol	100	91	100	100	100	81	93
2-Methyl-1-propanol	100	97	100	100	100	85	100
1-Pentanol	100	88	97	100	81	81	94
3-Methyl-1-butanol	100	100	97	100	83	86	90
Compounds added to solid media							
1-Hexanol	0 [c]	1.3	0	0	0	0	1.0
1-Nonanol	2.0	4.0	3.3	1.7	2.0	3.7	3.0
1-Decanol	4.0	4.0	4.0	2.7	3.7	4.0	4.0
1-Hendecanol	4.0	4.0	4.0	3.0	4.0	3.7	4.0

[a] Compound (0.1%) added to $NH_4H_2PO_4$-salt medium.
[b] % light transmittance, uninoculated blank = 100.
[c] Average of 3 readings (0 = no growth, 1 = slight growth, 2 = medium growth, 3 = good growth, 4 = abundant growth).

sine were not satisfactory N sources for *P. putrefaciens*.

When the amino acids were added to give 0.1 mg N per 10 ml of medium, cystine, methionine, and tryptophan did not support growth as well as aspartic acid, glycine, or proline. A similar observation has been reported for *P. fragi* (Ousley, 1957). When the amino acids were used as the sole source of both C and N, extent of growth was less than with added glucose. Under these conditions, glutamic acid and proline were superior in promoting growth. The activity of aspartic acid and glutamic acid is not surprising, since they are key intermediates in the N metabolism of bacteria and are associated with the tricarboxylic acid cycle. Also, in certain bacteria, a metabolic association exists between proline and glutamic acid (Vogel, 1953).

Albumin and gelatin were less effective than casein in promoting the growth of *P. fluorescens* and *P. fragi* (Ousley, 1957). It is unlikely that a difference in amino acid composition of the proteins is responsible for this observation. Subsequent experiments have shown that the proteolytic enzyme system of *P. fluorescens* attacked casein more readily than albumin (Hurley and Vanderzant, 1962).

A comparison of the utilization of C sources by strains of *P. fluorescens*, *P. putrefaciens* (Reinbold *et al.*, 1953b), and *P. fragi* (Ousley, 1957; Pereira and Morgan, 1957) shows that arabinose, glucose, and starch were utilized by all or a majority of the cultures of these 3 pseudomonads. Glucose was the only C source used by all cultures. Arabinose, xylose, fructose, galactose, glucose, mannose, trehalose, starch, glycerol, and erythritol were used by all or a majority of the cultures of *P. fluorescens* and *P. fragi*. Compounds involved in or associated with the tricarboxylic acid cycle, such as citrate, fumarate, malate, lactate, succinate, cis-aconitate, pyruvate, α-ketoglutarate, and isocitrate, were satisfactory C sources for many strains of these three pseudomonads. *P. putrefaciens* was definitely superior in utilizing naturally occurring fatty acids as the sole source of C. Propionic, butyric, valeric, lauric, myristic, palmitic, stearic, and oleic acid were utilized by *P. putrefaciens* but not by *P. fluorescens* or *P. fragi*. Most alcohols were not satisfactory as the sole source of C.

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Inactivation-Rate Studies on a Radiation-Resistant Spoilage Microorganism

III. Thermal Inactivation Rates in Beef[a, b]

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SUMMARY

The thermal resistance of *Micrococcus radiodurans* R_1, a spoilage bacterium highly resistant to ionizing radiation, was characterized. A modified "thermal-death-time tube" was used, with cells uniformly suspended in a raw meat purée reconstituted from freeze-dried and powdered beef that had been screened to remove pipette-plugging fibers and irradiated to eliminate viable aerobic organisms before use. Thermal death rate, unlike the radiation death rate, seemed to approximate an exponential form, as indicated by survival curves. A $D_{140} = 0.75$ and $z = 10.65$ describes the heat resistance in beef. Simple calculations suggested that if all parts of the samples of beef reached 150° F, the lower level of the "medium-done" range, a 1-min hold at that temperature should reduce viable numbers by a factor of 10^{-10} and a 2-min hold should reduce numbers by a factor of 10^{-20}.

INTRODUCTION

Micrococcus radiodurans, a nonsporing spoilage bacterium highly resistant to ionizing radiation and to ultraviolet light has been described (Duggan *et al.,* 1959, 1962a, b; Anderson *et al.,* 1961). This organism was isolated from spoiled meats that had been irradiated to 2 or 3 megarep.

The extreme resistance to radiation of this microorganism makes "commercial sterility" by irradiation difficult to obtain without adverse effects on flavor. The fact that these cells are nonsporing units raises fundamental questions about their resistance mechanism. It is also of practical interest in that the absence of spore formation suggests that other lethal agents may be used to inactivate these organisms. Heat is the most obvious and most common agent used in food preservation. Therefore, heat-survival curves in meats were considered basic to determination of a process for elimination of these organisms from the product.

Data (Drake *et al.,* 1961; Chiambalero *et al.,* 1959) on the storage of uncooked irradiated foods indicate that the normal food enzymes will have to be inactivated. Either a precooking or a blanching treatment before, during, or after irradiation will be necessary to prevent enzymatic deterioration of the food on storage. When such an enzyme inactivation treatment is established, it will be useful to have data to determine whether it might serve a double purpose by reducing these spoilage bacteria to a commercially safe level.

Toward this end, survival curves for the culture exhibiting the highest radiation resistance (Duggan *et al.,* 1962a) was determined at several temperatures in a beef substrate, and a thermal resistance curve was constructed.

MATERIALS AND METHODS

The raw puréed beef substrate was prepared as previously described (Duggan *et al.,* 1962a), *i.e.,*

[a] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, Q. M. Research and Engineering Command, U. S. Army, and has been assigned no. 2239 in a series of papers approved for publication. The views or conclusions are those of the authors, and not to be construed as necessarily reflecting the views or endorsements of the Department of Defense.

[b] Technical Paper 1594, Oregon Agricultural Experiment Station, Contribution of the Department of Microbiology.

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by screening out fibrous material from freeze-dried powdered meat, reconstituting to normal water content, and irradiating to 2 megarep (1.87 megarad) in sealed cans. No viable aerobic flora could be detected after this exposure, but the samples were stored frozen until used.

The R_1 cultures were grown as previously described (Duggan *et al.*, 1962a). Centrifuged cells were resuspended in 30 ml of 0.5% saline. This volume was mixed with 30 g of the irradiated beef purée described above, and the mixture could be pipetted easily to allow quantitative bacteriological studies. The inoculated meat (approx. $2\text{–}5 \times 10^8$ viable cells per ml) was refrigerated until used. Viable numbers did not decrease while the meat was in refrigerated storage.

Glass tubing (5 mm O.D., 1-mm wall thickness) was cut into 9-in. sections. After calibration and sterilization, these thermal death rate (TDR) tubes were filled to a 1-ml mark with the inoculated meat. The meat aliquots were centered in the TDR tubes, and the tubes were carefully sealed at both ends with sealing-wax. They were then placed in ice water until heat treated.

A number of TDR tubes were placed in a wire basket and tempered 3–5 min at 37°C before the lethal heating. After three tubes had been removed for the determination of initial numbers, the basket of tubes was lowered into a water bath held at the desired temperature, ± 0.5°F. Timing of the heat treatment began after the temperature of the substrate in the TDR tubes came up to bath temperature (in about 15 sec.). This was predetermined for each temperature by a thermocouple in a substrate-filled tube. Corrections were subsequently applied to the data to correct for lethality of "come-up" and "cooling" time. At definite time intervals, single tubes were removed into heavily chlorinated ice water. The brittle sealing-wax tips were chipped off and the meat sample was rinsed into dilution blanks for plating on TGY agar (tryptone 0.5%, yeast extract 0.1%, glucose 0.1%, agar 1.5%). Survivors, reported as the log of surviving numbers, were determined from the average values of five replicates.

The rate of temperature change in the TDR tubes during heating and cooling was determined with a thermocouple. Conduction errors from the thermocouple wire were admitted but could not be estimated.

A method suggested by C. R. Stumbo (personal communication, 1959) for thermal-death-*rate* corrections due to "come-up" and "cooling" time was used. This method is similar to that described by Annellis *et al.* (1954) for thermal-death-*time* corrections. A thermal-death-rate (TDR) curve was constructed on inverted semilog paper from the experimental data, and a straight line was fitted by the method of least squares. Lethal-rate values (the reciprocal of the thermal death time) were calculated for several points and a curve was made. (D-value is the reciprocal of the slope of the survival curve and represents the time in minutes to reduce numbers by 90%.) On the basis of these data, a plot was made of lethal-rate versus time on the "come-up" and on the "cooling" curves. A smooth curve was fitted to this lethal-rate curve. The area under the lethal-rate curve represents the inactivation occurring during heating and cooling of the tubes and acquires a numerical value when compared to the area under a curve having a known inactivation effect. Such a curve in the form of a rectangle was constructed on the lethal-rate-curve coordinates with the ordinate equal to the lethal rate at the given bath temperature. The abscissa corresponded to 1 min of heating time. The area of this rectangle represents inactivation occurring during a treatment in which the sample is instantaneously heated to bath temperature, held there for 1 min, and instantaneously cooled. The ratio of the areas under the lethal-rate curves for "come-up" and "cooling," to the area in the rectangle described, provided the time in minutes to be added to the timed exposure at the temperature used.

To summarize, the corrected exposure time is the sum of the timed exposure interval plus the time-equivalent, at exposure temperature, of the "come-up" and "cooling-down" times.

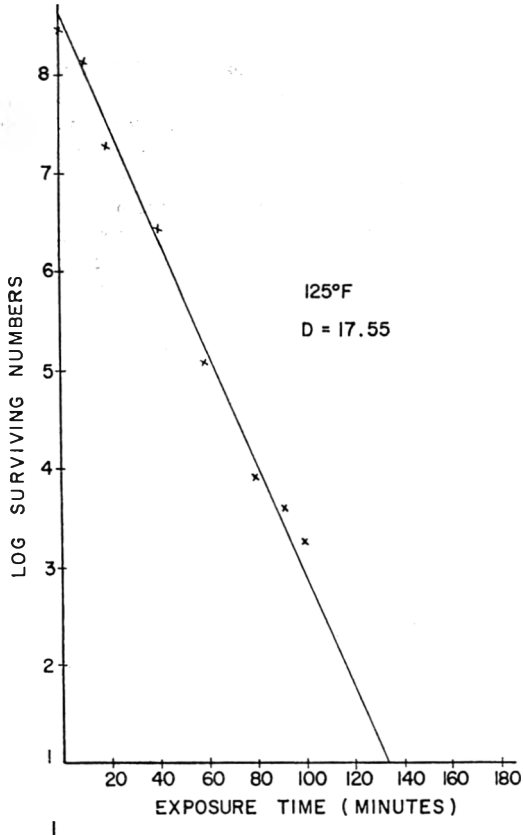
Plots were made on semilog paper of surviving numbers versus corrected exposure time for each exposure temperature. Straight lines were fitted by means of the least-squares method. The inverse of the slopes of the lines yielded the D-value at each temperature. The thermal resistance curve was constructed on semilog paper by plotting these derived D-values versus the exposure temperatures and fitting a straight line by means of the least-squares method. The inverse of the slope of the thermal resistance curve yielded the z-value.

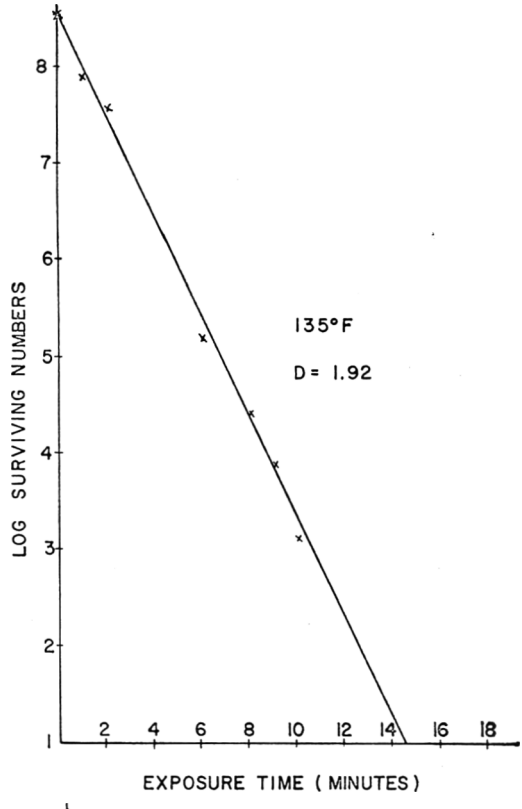
RESULTS

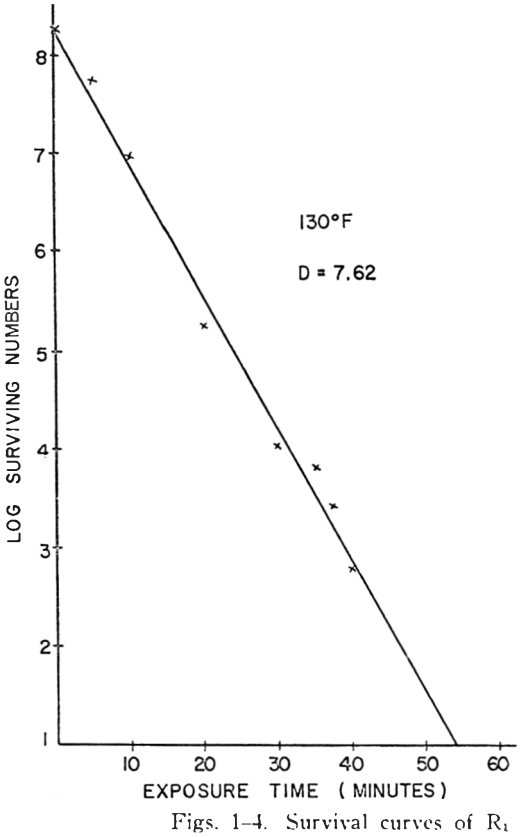
The R_1 culture of *Micrococcus radiodurans* appears to be inactivated in the meat substrate at an exponential rate (Fig. 1–4), with D-values, as determined by least-squared method, as follows:

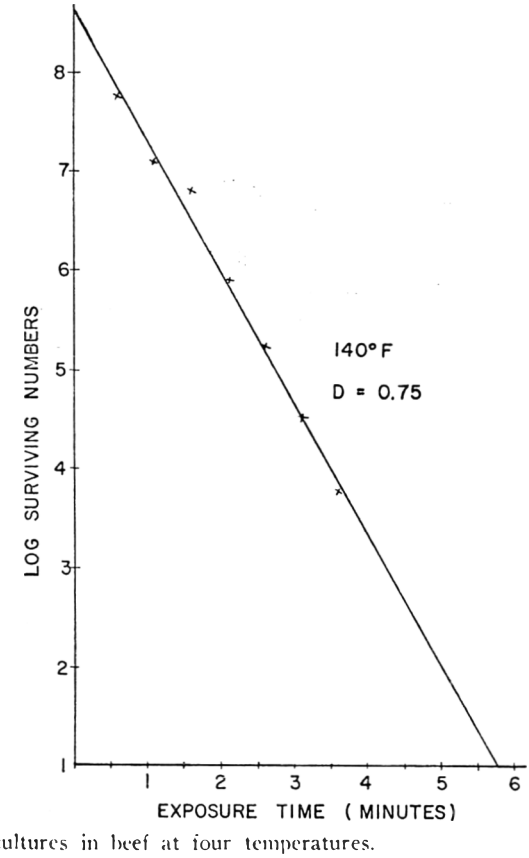
Temperature (°F)	D value
125	17.55
130	7.62
135	1.92
140	0.75

The thermal resistance curve (Fig. 5) reveals a z-value of 10.65 as determined by least-squares method.









Figs. 1–4. Survival curves of R_1 cultures in beef at four temperatures.

DISCUSSION

Heat destruction of the R_1 culture in beef approximates an exponential rate. This is of interest since survival curves obtained after exposure to gamma radiation and to germicidal ultraviolet light appeared to be sigmoidal in nature (Duggan *et al.*, 1959, 1962a). One of the theories considered in attempting to explain the sigmoidal radiation survival curves (and to account for part of the radiation resistance), was that the cells were multinuclear or multicellular in basic makeup. However, if the organism were truly multinuclear or if a multicellular phenomenon were involved, it is natural to expect that the heat survival curves would also be of sigmoidal plot. Since sigmoidal survival curves were not observed on thermal treatment, the multitarget proposal would seem to be unsupported. This assumption, however, presupposes that the same essential structure is involved in inactivation by heat and by radiation.

The z-value near 11 falls within the usual range of 10 to 14 (Foster *et al.*, 1957) or 8 to 20 (Olson *et al.*, 1952) observed for vegetative cells.

Both psychrophilic and mesophilic strains of *Micrococcus radiodurans* have been isolated, and all strains are apparently capable of at least limited growth in canned irradiated meats. Their potential for spoilage must be limited, then, whether or not they are refrigerated.

Determination of a thermal process designed to result in a "commercially sterile" meat according to canning-industry standards would involve, first of all, agreement as to the maximum possible levels of spoilage that would be tolerated. For pathogens, the maximum possible spoilage level has been set by the canning industry at levels on the order of one in 10^{12} samples (Gillespy, 1951; Townsend *et al.*, 1954). For nonpathogenic thermophilic spoilage organisms, maximum possible spoilage tolerances are often on the order of one in 10^2 or in 10^3 samples ($F = 5\ D$) (Townsend *et al.*, 1954) at the rarely attained temperatures favorable to thermophiles. Non-pathogenic mesophilic spoilage is limited to a maximum possible spoilage tolerance in the range of one in 10^3 to one in 10^5 samples ($F = 5\ D$ to $7\ D$) (Townsend *et al.*, 1954). Using the data collected here, certain calculations can be made if we assume a maximum initial count in hamburger of about 2,500 viable cells per gram (approximately 10^6 per pound), and a maximum possible spoilage level of one in 10,000 samples (for example) of one-pound size. These figures would indicate that an over-all reduction of 10^{-10} from the initial load is required—a reduction over 10 log cycles. A maximum possible spoilage level of one in 10^{12} samples would require an over-all reduction of 10^{-18}.

Transversing 10 log cycles involves exposing the samples to 10 D-values (in minutes) at the temperature used. At 140°F, the samples would be subjected to the equivalent (Stumbo, 1953) of 10×0.7535, or about 7.5 min. Similar figures for a reduction of 10^{-18} at 140°F would be 18×0.75, or about 13 min.

It would seem reasonable to accept as "commercially sterile" with respect to this micrococcus, any meat all parts of which had been held for either 1 or 2 min (depending on the spoilage level adopted) at a minimum temperature of 150°F (where $D_{150°}$ would be less than 0.1 min). Holding for 1 min at this temperature would reduce initial numbers by a factor of 10^{-10}, whereas holding for 2 min would reduce numbers by a factor of at least 10^{-20}.

The required time of exposure (of a sample) to a lethal temperature depends on the size, shape, and thermal diffusivity (Ball and Olson, 1957) of the sample, as well as the nature of the container, form of heat, and other factors as they influence the total destructive effect of the "integrated heat value" (Stumbo, 1953). Therefore, no attempt will be made to estimate exposure times in any container. One could calculate safe processes for any sample, however, using the rate-of-destruction curves presented here, after heat penetration data had been accumulated for the sample.

If both radiation and heat are to be used in radiation preservation, the influence of each agent should be assessed in terms of its effect on the numerical destruction required of the other agent. Pre-irradiation would

reduce initial numbers present before heat treatment, and would result in a lowering of thermal process necessary to reach the same level of spoilage limitation. Post-heating radiation would likewise reduce the survivors of thermal processes to a point where considerably less thermal destruction would be required. In addition, it has been shown that heat treatment reduces the resistance of this organism to subsequent irradiation (Duggan *et al.*, 1962b). It has also been shown that irradiation at higher temperatures (40 and 50°C) is more effective in activating this organism than irradiation at temperatures below 40°C. It may be of some advantage, then, to use radiation and heat treatment concomitantly in attempting to limit spoilage due to this organism.

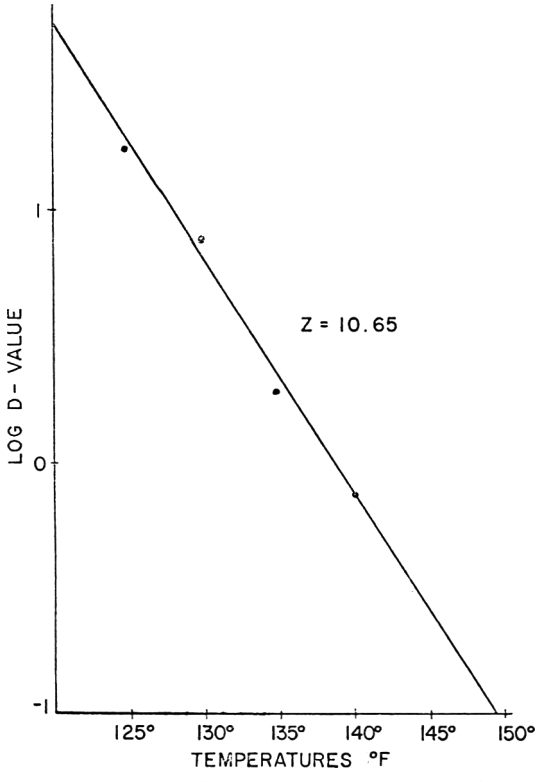


Fig. 5. Thermal resistance curve of R_1 culture in beef.

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Direct Microscopic Technique to Detect Viable Yeast Cells in Pasteurized Orange Drink

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(Manuscript received July 23, 1962)

SUMMARY

Biological spoilage of non-carbonated orange drink is caused mainly by yeasts that are not inhibited by preservatives at permitted levels. A stain that distinguishes living from dead yeast cells was used to develop a technique that detected yeast growth within 24–36 hr of the addition of one viable yeast cell per 190 ml of pasteurized orange drink.

INTRODUCTION

The aim was to develop a rapid test for the routine control of biological defects in a non-carbonated pasteurized orange drink containing 5% of pure orange juice. The product has a pH of 3.5, titratable acidity as citric 0.25%, and specific gravity 1.039, and is flavored with an emulsion of cold-pressed orange oil. It is pasteurized 20 sec at 170°F, cooled to 80°F, and packed in polyethylene-lined "Tetrapak" cartons with a capacity of one-third imperial pint (190 ml). The product is stored at ambient temperatures ranging from 40 to 100°F in shops and canteens, for periods of 1–4 weeks.

Commercial experience and laboratory tests indicate that the pH and composition of the drink inhibits most bacteria, and that lack of head-space in the pack appears to control the growth of most of the molds. However, a yeast isolated from orange juice grows readily in the drink over a wide range of temperatures. No specific identification of this yeast has been attempted, but information and photomicrographs supplied by the Low Temperature Research Station, Cambridge University (Ingram, 1960a), indicate that it may be a strain of *Saccharomyces acidifaciens* or *S. elegans*.

Australian Health Regulations limit the use of preservatives in "Summer or Temperance Drinks" to 0.006% SO_2 or 0.02% benzoic acid, or proportionate mixtures of the two substances. These preservatives, and sorbic acid at 0.02%, have been tried against the yeast, but have shown no significant inhibiting effect. These negative results agree with those reported by Ingram (1959) on fermentation of squashes containing 0.06% benzoate, and by Smith *et al.* (1962), who found that some yeasts utilized up to 34% of the sugar in apple juice (pH 4.0) containing 0.025% of sorbate or benzoate. In the light of these facts, it was decided to rely on efficient pasteurization of our orange drink for maintenance of shelf-life, and to concentrate, in laboratory control work, on developing a test that would detect minimal initial yeast infection as soon as possible after the manufacture of each batch.

EXPERIMENTAL METHODS

Initial experiments. Plating of 1 ml of orange drink on potato-dextrose agar, buffered yeast extract, etc., regularly failed to detect yeast in samples that had been artificially infected and subsequently became fermented. Similar failures were experienced when 10-ml samples were centrifuged and the deposits plated and incubated.

Micro-filtration was found to be useful in detecting infection once a reasonably large number of cells were present. Unfortunately, the maximum amount of orange drink that would go through a 3.5-sq-cm membrane filter was 3 ml, and direct microscopic examination of the filters often failed to reveal viable cells in samples that ultimately showed fermentation.

Attempts were also made to apply the direct microscopic technique of Stevens and Manchester (1944) and other methods involving staining and examining 0.01 ml with an oil immersion lens. This work not only failed in most instances to

detect yeast in artificially infected samples, but when cells were seen it was not possible to say whether they were viable cells or some that had been destroyed by pasteurization.

The reason for these failures became clear when further work demonstrated that the addition of only one viable yeast cell per 190-ml sample of sterile orange drink was sufficient to cause vigorous growth and fermentation within 4–8 days at room temperature. It now became obvious that a yeast-detection test was required that used the entire contents of one pack (190 ml) as the test sample, and could detect an initial infection of 1 viable cell per 190 ml.

Microscopic examination of yeast cultures in wet mounts had shown that most of the cells settled to the bottom of the liquid on the slide within 1–2 min. This observation led to a series of experiments with 190-ml samples, as follows: The entire contents of the one-third-pint sample pack were poured into a separating funnel (capacity 250 ml) followed by 20 ml of 10% yeast extract, then inoculated with one viable budding yeast cell. The outlet tube at the base of the funnel was suitably protected from airborne contamination (see Fig. 1), then the funnel and contents were incubated 16–20 hr at 26°C. Then a 1-ml sample was run from the base of the funnel and plated on buffered yeast extract agar. The plate was then incubated at 26°C, and usually showed one or more yeast colonies after 48 hr of incubation. This work proved that an initial infection of only one active cell per 190 ml would result in one or more yeast cells per ml at the base of the funnel after 16–20 hr. The next problem was to find a rapid method of proving their presence without waiting a further 48 hr for colonies to show on the plates. It was realized that, if a stain could be found that clearly distinguished between living and dead yeast cells, then 10 ml could be taken from the base of the funnel at 16–20 hr and centrifuged, and the deposit examined microscopically to detect any viable yeast cells present.

A distinguishing stain. A number of recommended stains (Ketterer, 1956) were tried, and the best was that described by Townsend and Lindegren (1953). Unfortunately, their technique required that the cells be washed in distilled water prior to staining, which was virtually impossible with the small number of cells involved. Ingram (1960b) suggested that a stain described by Wolford (1953) should be tried. This stain was developed primarily to improve the counting of microbial cells in concentrated orange juice by creating more distinction between the cells (dark blue) and the background (pink to purple) than was obtained with other stains. However, Wolford did make reference to a distinction between viable and dead cells: "In some instances, when stains were made of juice containing actively growing micro-organisms, the microbial cells have appeared to be only lightly stained by the methylene blue. This result gives the staining method a differentiating property, as it gives some degree of distinction between actively respiring and inactive or dead cells."

Wolford's claim was found to be very modest when the stain was used on our yeast cultures. Pasteurized cultures showed 100% of the cells to be stained deep red to reddish purple, whereas in actively growing young cultures that had not been heated, the cells remained unstained. Older cultures showed some stained cells among clumps of unstained living cells. Mixtures containing known proportions of pasteurized and unpasteurized cultures showed the expected proportions of stained and unstained cells (see Figs. 2, 3). The apparent variation in the appearance of the living cells in Fig. 3 is due to variations in cell density, not to the presence of any stain. Wolford's stain was a development of Gray's (1943) methylene blue–

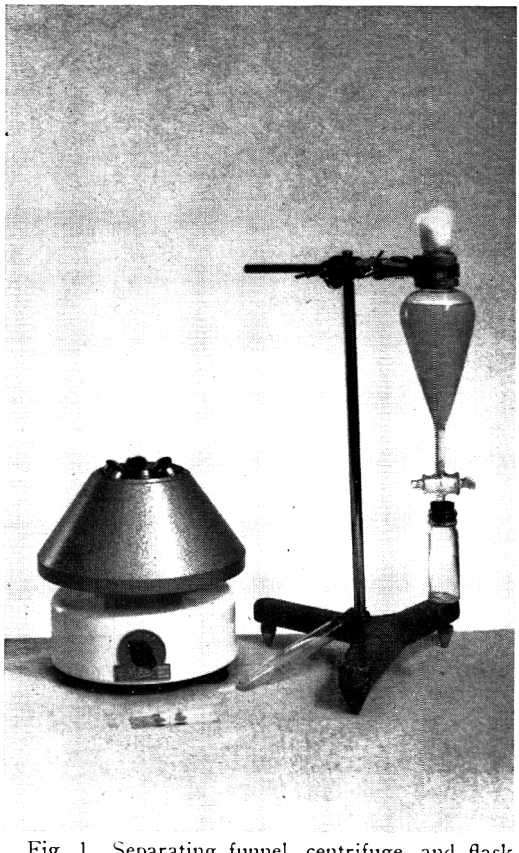

Fig. 1. Separating funnel, centrifuge, and flask with centrifuged deposit.

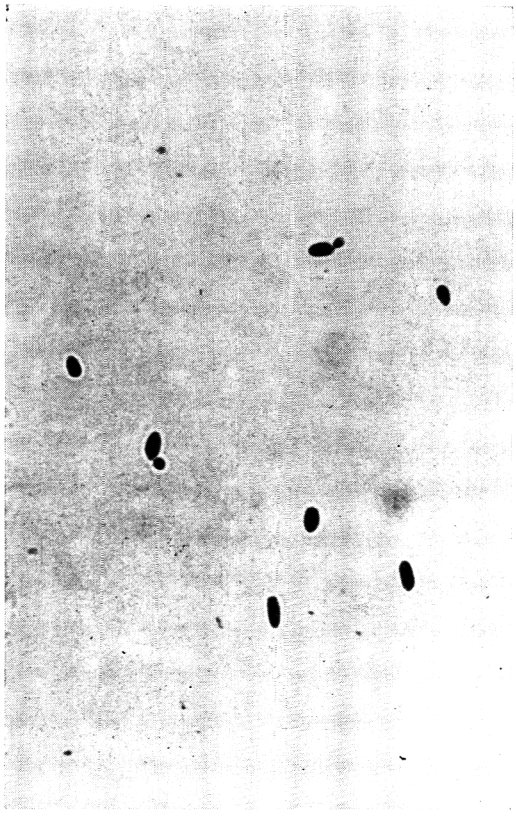

Fig. 2. Dead cells in a pasteurized culture, deeply stained. 600× wet mount.

basic fuchsin and North's (1945) aniline oil-methylene blue stain, and was prepared as shown in the Appendix.

The new microscopic yeast-detection test. Application of the Wolford stain to the initial experimental work previously described has resulted in development of the following test, which has detected the presence of yeast in pasteurized non-carbonated orange drink within 24–36 hr of the addition of one viable yeast cell per 190-ml sample.

1) Pour the contents of a ⅓-pint pack (190 ml) into a sterile 250-ml separating funnel (Fig. 1) using normal aseptic methods.

2) Add 20 ml of 10% yeast extract.

3) Invert funnel to mix well, then incubate 16–20 hr at 26°C. Invert funnel several times during incubation if convenient, but do not disturb the sample in the last 3–4 hr of incubation.

4) Remove funnel carefully from the incubator and run 10 ml from the base, through a sterile 200-mesh sieve, into a centrifuging flask.

5) Centrifuge 15–20 min at 5000 rpm.

6) Pour off the supernatant, leaving 1–2 drops of liquid in the flask. Mix the deposit and the liquid with a sterile platinum loop, then incubate for 6-8 hr.

7) Place 0.01 ml of deposit on a glass slide, add 0.01 ml of Wolford stain, and mix with the platinum loop, using a rotary motion and spreading the mixture over an area of about 1 sq cm.

8) Examine microscopically as a wet mount with 8× eyepiece and 10× lens. Examine the entire area of the sample before it dries. If an unstained yeast cell is seen, switch to a 40× lens and check for morphology and lack of staining. Dead cells will appear deep red to reddish purple.

9) If no cells are seen, incubate the centrifuging flask 12 or 24 hr further, and re-examine. No subsequent growth has been observed to date in any sample that showed no viable cells after 40 hours from the time the funnel was incubated.

Table 1 shows the time required with the new technique to detect viable yeast cells in artificially infected samples.

When cultures of the yeast used were developed from a single cell with orange drink and yeast

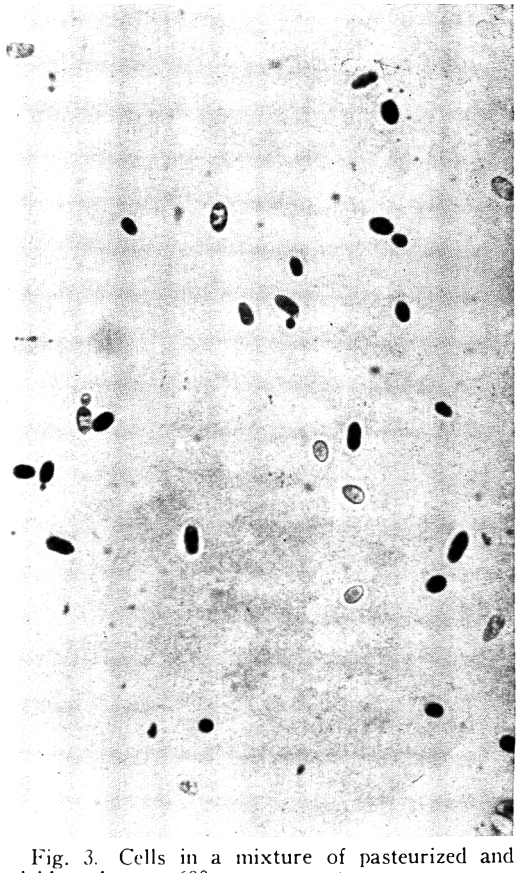

Fig. 3. Cells in a mixture of pasteurized and viable cultures. 600× wet mount.

Table 1. Time required to detect viable yeast cells in artificially infected samples.

No. of samples	Cells added/190 ml		Unstained budding cells observed at (hr)
	Budding	Non-budding	
2	2	18–20	16
3	5	...	16–20
3	1	...	24
2	1	...	30
7	1	...	36–40
4	...	2	36–40
3	...	1	NVC [a]
2	1	...	NVC
2	...	2	NVC

[a] NVC, no viable cells observed after 10 days of incubation.

extract as the medium, some of the resultant cells were only ½–⅓ the size of the parent cell (see Fig. 4). These small cells were actively budding and apparently mature. When normal-sized cells were plated on yeast extract-agar medium, some colonies were composed almost entirely of small cells, interspersed with occasional full-sized cells. When the small cells were grown in orange drink without enrichment with yeast extract, the resultant cells were of normal size. In examination of centrifuged deposits, therefore, a careful watch was kept for the smaller cells, which sometimes appeared sooner than cells of normal size.

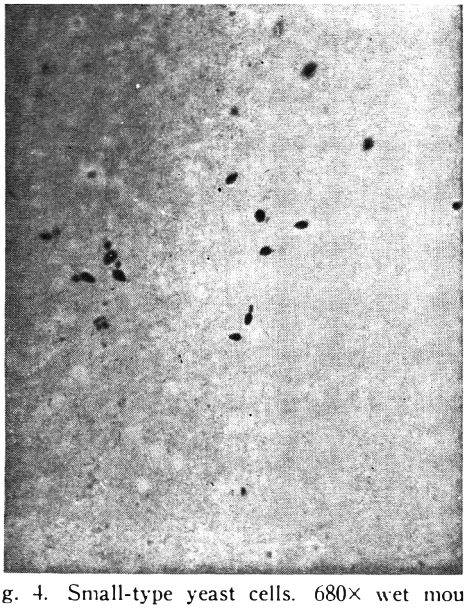

Fig. 4. Small-type yeast cells. 680× wet mount.

APPENDIX

The Wolford stain is prepared as follows:

Solution A. (North's stain). Dissolve 3 ml aniline oil in 10 ml 95% ethanol, add 1.5 ml concentrated HCl with stirring, add 30 ml saturated alcoholic solution of methylene blue, then add 55.5 ml distilled water.

Solution B. Dissolve 1 g of basic fuchsin in 100 ml of 95% ethanol.

The Wolford stain is prepared by adding 10 ml of Solution A and 2 ml of Solution B to 88 ml of distilled water. The mixed stain should be filtered before use and held under refrigeration. It should be discarded after 3–4 weeks.

DISCUSSION

Liquid foods are usually distributed to retail outlets within 1–2 days of manufacture, so rapid appraisal of the probable shelf-life of each day's production is very desirable. The foregoing work assists in meeting this requirement with orange drink. The technique described may have an application in the laboratory testing of pasteurized fruit juices, squashes, wines, beer, and other liquid foods in which fermentation by yeasts is an important factor.

ACKNOWLEDGMENTS

The author expresses sincere appreciation for helpful advice given by Dr. J. H. B. Christian, Division of Food Preservation, C.S.I.R.O., and Dr. M. Ingram, University of Cambridge, and thanks P. R. Maguire for the photomicrographs of yeast cells.

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Growth of Psychrophiles. I. Lipid Changes in Relation to Growth-Temperature Reductions[a]

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(Manuscript received August 27, 1962)

SUMMARY

A monomolecular surface-film method was used to evaluate lipid changes in bacteria isolated from refrigerated poultry meat. Changes in cell lipids were studied as functions of reduced growth temperature and growth in the presence of low concentrations of chlortetracycline. Temperature reduction did not cause lipid changes in representative psychrophilic bacteria but did cause profound changes in a gram-positive rod having a lesser ability to grow at 4°C. Growth in the presence of CTC caused lipid increases in all strains.

INTRODUCTION

Investigations dealing with bacterial lipids have been confined, for the most part, to problems of lipid chemistry, lipid storage, or cellular organization. Problems of lipid chemistry have been associated primarily with the Mycobacteria, other bacterial species (Salton, 1960, 1962), as well as protozoa and fungi (Hutner and Holz, 1962). Studies of lipid storage have been mostly confined to the yeasts. Recent interest, however, has increased concerning poly-β-hydroxybutyric acid and whether it should be considered, along with triglycerides and glycogen, as a major reserve material in many bacteria (Gunsalus and Stainer, 1960).

Effect of environment on chemical changes and on increases in fat inclusions in microbial cells has been considered primarily from the make-up of the nutrient medium in which the cells have been grown (Hutner and Holz, 1962). Herbert (1961), in a review paper, recently made several suggestions concerning future work on the chemical composition of bacterial cells. He pointed out a need for consideration of the external environment and a need for more work to better relate external environment to the composition and size of the cell.

Although intact-cell and cell-wall structure of gram-negative or gram-positive bacteria has been investigated with great vigor, studies concerned specifically with lipids and lipid changes are not numerous. This is especially true with regard to environmentally induced changes. The relative amounts of lipids in species have been reported by Salton (1953, 1962), Salton and Horne (1951), and Cummins and Harris (1955). Differences in the saturation of bacterial lipids as a function of temperature were shown by Graughran (1947). Graughran's report stated that as the growth-temperature for mesophiles was increased the degree of saturation and the quantity of cellular lipids decreased. The papers presented at the eleventh symposium of the Society for General Microbiology (1961) only tend to emphasize the paucity of information in this area.

In light of the structural differences between the gram-negative and gram-positive bacteria and the relation of temperature to growth rate, a study was suggested that would relate lipid changes to the environment of psychrophiles. Ingraham and Bailey (1959) thought that differences in growth rates between psychrophilic and mesophilic

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bacteria might be due to changes in cellular organization. Such a change could, conceivably, be reflected in changes in the lipids of the bacterial cell.

In the present study, cell lipids and growth rates were measured in isolates from refrigerated poultry meat that had undergone a typical spoilage at 4°C. Effects of temperature reductions and growth in the presence of chlortetracycline (Aureomycin) were evaluated. This antibiotic was chosen since it is permitted as an extender of shelf-life of fresh poultry meats.

EXPERIMENTAL METHODS

Bacterial strains. The species used were isolated from poultry meat that had been held 8 days at 4°C. Selection and characterization of the isolates were continued until three cultures exhibiting different low-temperature growth patterns were chosen for study (Wells, 1961): *Pseudomonas fluorescens*, *Achromobacter guttatus*, and *Brevibacterium linens*. The *Brevibacterium* species was included for comparison of reactions of gram-negative and gram-positive rods. The *Pseudomonas-Achromobacter* species were chosen because of the predominance of these types in poultry meat spoilage (Ayres *et al.*, 1950).

Culture preparation. All cells were grown on trypticase-yeast extract agar (2% trypticase, 0.5% yeast extract). When growth in the presence of chlortetracycline (CTC) was desired, the sterilized medium was allowed to cool to 50°C and a filter-sterilized solution of CTC was added to a final concentration of 1 μg/ml. Cells were harvested during the exponential growth phase by washing the cell-mass from the agar surfaces with sterile distilled water. After breaking the cell clumps by shaking with glass beads, the suspensions were filtered through double-layered cheesecloth and centrifuged. The packed cells were suspended in water and spun again. Washing in this manner was repeated four times.

Finally, the cells were suspended in enough water to make a thick slurry. This slurry was frozen as a shell in a 60-ml vial and dried from the frozen state under vacuum (Virtis freeze dryer).

Lipid determination. A rapid and reproducible method for determining small quantities of lipids was available in the monomolecular surface-film method described by Bergquist and Wells (1956). The method, as applied to bacterial cell lipids, was changed somewhat in that it was not found necessary to add the alkali solution before extraction; precipitation and coagulation of native proteins was not a problem, as it was with egg albumen, for which the method was originally developed. The procedure for determination of bacterial lipids was as follows: approximately 150 mg of lyophilized cells were weighed into a covered 50-ml Erlenmeyer flask. Ten ml of 6*N* HCl was added, and the suspension heated 2 hours at 100°C. After the hydrolysate cooled to room temperature it was transferred to a 4-oz oil-sample bottle. Extraction of lipid was accomplished by addition of 50 ml of a 1:1 mixture of ethyl ether-petroleum ether, shaking the ether-hydrolysate mixture 100 times through a 1-foot arc, then allowing the mixture to stand until the solvent separated, which required about 2 minutes. Twenty-five ml of the ether layer was then transferred to an aluminum evaporating dish, and the ether was evaporated from the dish on a steam bath. Thereafter, the procedure of Bergquist and Wells (1956) was followed. The reader is referred to the original description of material and methods for calculation of lipid. The spreading coefficient was determined from extracts of whole cells. The averages presented in the tables were determined from nine analyses.

RESULTS

Pseudomonas fluorescens grown at 25°C was found to have a lipid content of 12.2% based on dry cell weight. Growth at 4°C did not appear to induce changes in this relative proportion (Table 1). *Achromobacter guttatus* was also found to be uninfluenced by changes in growth temperature in regard to the relative abundance of lipid material. The most noticeable difference between these two organisms appeared to be a normally higher lipid level in *A. guttatus*.

Table 1. Percent lipid in psychrophilic bacteria grown at different temperatures.

Culture	Incubation temperature	
	4°C	25°C
Pseudomonas fluorescens	11.8%	12.2%
Achromobacter guttatus	18.4%	18.5%
Brevibacterium linens	16.7%	7.2%

Brevibacterium linens responded differently to temperature reduction. At 25°C it had a dry cell lipid content (7.2%) less than that of either *P. fluorescens* or *A. guttatus*. When grown at 4°C, however, it responded by increasing its lipid fraction to a quantity intermediate to that of the other two test strains.

Analysis of all three test strains, grown in the presence of CTC, indicated that exponential-phase cells had responded to the antibiotic through increased amounts of lipid in the cells (Table 2).

Table 2. Lipid changes in psychrophilic bacteria in the presence of chlortetracycline.

Culture	Incubation temperature	
	4°C	25°C
	1 ppm CTC	1 ppm CTC
Pseudomonas fluorescens	16.3%	14.3%
Achromobacter guttatus	21.1%	20.0%

Chlortetracycline at 1 μg/ml brought about a lipid increase of 17.2% in *P. fluorescens* when the cells were grown at 25°C. The addition of CTC to the growth medium of this strain, grown at 4°C, brought about an increase in lipid amounting to 38.1%.

As previously noted, reduction of the growth temperature from 25°C to 4°C had little effect on lipid changes in the cells of *P. fluorescens*; however, with CTC present, at both 25 and 4°C the amount of lipid in cells harvested at the lower temperature increased nearly 14%.

CTC at 1 μg/ml in the medium caused similar responses in *A. guttatus*, though not to the same degree as observed with *P. fluorescens*. The antibiotic added at 25°C increased the lipid level slightly (8%). CTC added at 4°C resulted in a greater lipid increase than CTC added at 25°C.

The effects of CTC on *B. linens* could not be determined, because this antibiotic, added to the growth medium, suppressed cell growth for over one month. When growth did occur, it was scant and insufficient for analysis.

To determine just where the upper limit of response to antibiotic lay, *P. fluorescens* was grown at 25°C in the presence of several concentrations of CTC. Fig. 1 shows the responses. With amounts in excess of 5 μg/ml, the change in percent lipid was negligible.

DISCUSSION

The primary objective of the work was to determine whether the relative proportion of lipids in poultry spoilage bacteria could be changed by environmentally induced factors. Further, if such changes did occur, could they be related to the ability of bacteria to grow at temperatures used for storage of fresh poultry meats?

In view of the constancy of the lipid content in the two active psychrophilic strains and the lipid increase resulting from lowering the growth temperature for the *Brevibacterium* strain, the data suggest that cell lipid change is either involved in or does determine whether a cell will grow at temperatures commonly used to refrigerate fresh meats.

P. fluorescens and *A. guttatus* are representative of the organisms predominating on poultry meat stored at 4°C (Ayres *et al.*, 1950; Walker and Ayres, 1956; Ng *et al.*, 1957). *Brevibacterium linens* is representative of the types that are evident at the beginning of storage but decline during storage.

Chlortetracycline used to extend the shelf life of fresh poultry meat gives rise to populations exhibiting increased lipids. With an increase in lipid, especially if in the cell wall, an organism could experience impaired absorption. Impaired absorption could affect rates of enzymatic action (in addition to temperature reduction) since building materials entering the cell would become a limiting factor.

However attractive this suggestion may be, much work yet needs to be done before it can be finally proven. The cells used for the lipid assays were taken during the exponential growth phase. This introduced a variable. Although the growth phase was the same for all cells, the time required to reach that phase differed. With temperature as the environmental factor, no increases in

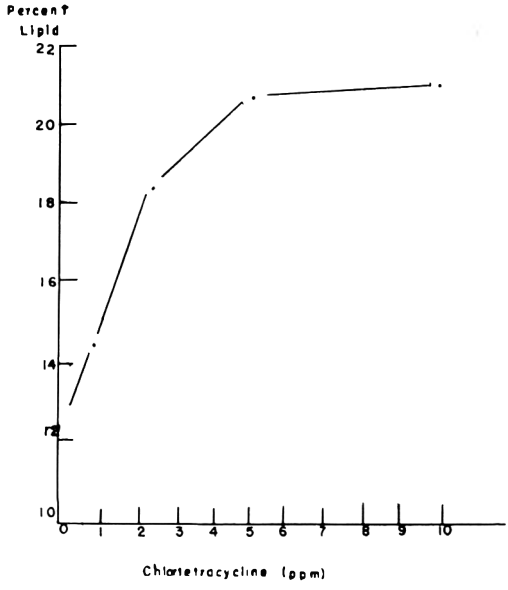

Fig. 1. Effect of level of exposure to chlortetracycline on percent lipid in cells of *Pseudomonas fluorescens* (at 25°C).

lipid in the psychrophiles were noted, and we may assume that temperature reduction had little selective action. With antibiotic as the environmental factor, however, lipid increases were noted. The problem that arose—at the moment unresolved—was whether the increase in the lipid fraction was a consequence of the selection of antibiotic-resistant strains (where high lipid is a factor in the resistance) or whether the increases were the result of physiological adaptions, e.g., degree of saturation of the lipid.

The authors feel that we are not simply selecting resistant strains where the resistance is a function only of the higher lipid content. Fig. 1 shows that as antibiotic concentration increased, lipid increased until the level of 5 μg/ml was reached. From this point upward, to 10 μg/ml, little increase in lipid was obtained.

The lag phase was considerably longer, however, in cells grown in the presence of antibiotic at 10 μg/ml than at 5 μg/ml.

Since the size of the inoculum was equal in each case, it would appear that the cells challenged by 10 μg/ml antibiotic were under some added stress. The lipid content was no greater in cells influenced by the higher level than in those influenced by the lower antibiotic level. It is our feeling that the lipid increases due to the presence of CTC noted in this study are a physiological response although this response could have a bearing on antibiotic resistance through changes in cellular absorptive characteristics followed by a different sequence of biosynthetic functions.

Other investigators have shown that growth rates may differ between large and small colonies within a strain (Stokes and Bayne, 1957; Clowes and Rowley, 1955). These investigators postulated that rate differences may be related to permeability of the cells. A decreasing ability to absorb nutrients could also partially explain the differences in growth rates of bacteria at low temperatures. Cell lipids and changes (increases or no response) induced by lowered growth temperatures or the presence of CTC could have an influence on permeability rates.

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Maple Sirup. XXI. The Effect of Temperature and Formaldehyde on the Growth of Pseudomonas geniculata in Maple Sap

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SUMMARY

The growth of *Pseudomonas geniculata* in sterile maple sap in the presence and absence of 5 ppm formaldehyde at several temperatures was investigated. At 27°C, the growth of inocula as low as 10^3 cells/ml was rapid, and the effect of formaldehyde, if any, was negligible. At 7°C, the temperature slowed the growth of the bacteria, particularly in cultures inoculated with 10^3 or 10^4 cells/ml. Formaldehyde further inhibited the cultures, initially causing a decrease in the number of viable organisms and lengthening the lag period before growth began again. This was particularly noticeable in the cultures with the smaller inocula.

Tapholes for the collection of sap from maple trees are liable to infection by adventitious organisms. Naghski and Willits (1955) demonstrated that "prematurely dried-up" tapholes, a condition in which sap flow diminishes or ceases completely, are heavily contaminated with bacteria. Sheneman *et al.* (1959) found a high degree of correlation between the occurrence of large populations of bacteria early in the sap season and low yields of sap. Since the premature stoppage of sap flow results in serious economic losses of the maple sirup crop, an intensive investigation was carried out to find inhibitors for bacterial growth in sap. Paraformaldehyde (a polymer of formaldehyde) has proven the most promising compound in controlling the growth of microorganisms in a taphole (Sheneman *et al.*, 1959; Costilow *et al.*, 1962).

Formaldehyde, the active ingredient of paraformaldehyde, is a well known bacterial inhibitor. There is, however, a great deal of variation in the reports of the action of formaldehyde on microorganisms, due, perhaps, to the differences in the experimental conditions and organisms used. McCulloch and Fuller (1941) found that 1% formalin inhibited the growth of *Erysipelothrix rhusiopathiae* for only 24 hours after 10 minutes of exposure, and it required 30 minutes of exposure for a 4% solution of formalin to kill the organisms. Other studies (McCulloch, 1945) also used concentrations of formaldehyde as great as 5% to prevent the growth of a variety of organisms. Temperature, too, affects the activity of formaldehyde; McCulloch and Costigan (1936) reported that a solution of formaldehyde having germicidal activity at 40°C may be of questionable value at 20°C and almost inert below 10°C.

Pseudomonas geniculata is the organism most frequently isolated from maple sap. Costilow *et al.* (1962), in a report issued while this paper was in preparation, indicated that the growth of this organism, at an inoculum level of 10^4 organisms/ml, is completely inhibited by 30 ppm formaldehyde, and some inhibition occurs at lower concentrations.

The quantity of dissolved formaldehyde in sap collected from tapholes containing paraformaldehyde pellets is 5 ppm or less in approximately 92% of the saps studied (actually about 70% of the saps contained 1 ppm or less of formeldehyde) (Costilow

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et al., 1962). The temperature conditions under which maple sap is collected are usually below 10°C. Thus, according to the literature, the conditions in which paraformaldehyde is used in maple tapholes should not be conducive to inhibition of bacterial growth in the maple sap after it leaves the taphole.

This report describes a limited study of the effect of the specific concentration of formaldehyde most likely to be encountered in the field, namely 5 ppm, on the growth of *Pseudomonas geniculata* at 7 and 27°C. It was important that this concentration of formaldehyde be studied since it is anticipated that maple sap will eventually be used in controlled fermentations to yield sirups of desired properties.

MATERIALS AND METHODS

Ps. geniculata, strain 4, was isolated from maple sap (Naghski and Willits, 1955) and has been shown to produce maple flavor precursors in sterile sap through controlled fermentation. The organism was cultured in a medium containing maple sap sterilized by autoclaving 15 min at 121°C and decanted under aseptic conditions from the heat-precipitable material. To this sap was added a quantity of sterile Difco (no endorsement implied) yeast extract solution to give a final concentration of 0.5% in the medium. After incubation at room temperature (25°C) for approximately 48 hr the cells were collected by centrifugation and suspended in sterile distilled water. Appropriate dilutions were made in sterile distilled water.

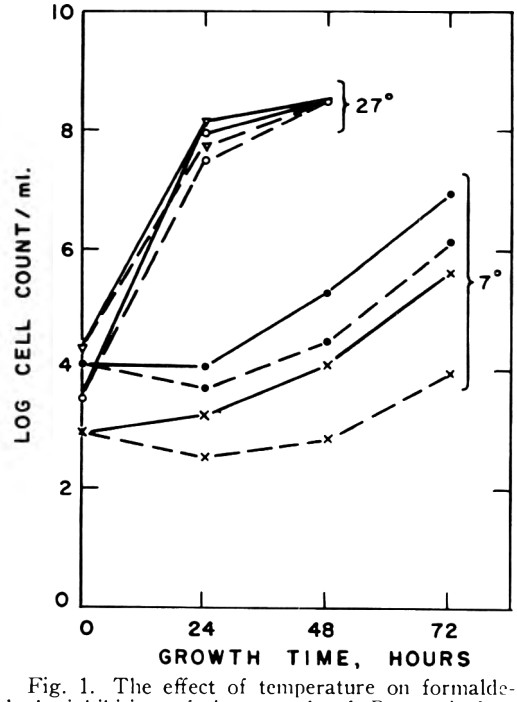

Fig. 1. The effect of temperature on formaldehyde inhibition of the growth of *Ps. geniculata*. Control (no formaldehyde), ———; 5 ppm formaldehyde, — — — —.

Erlenmeyer flasks, of 500 ml capacity, were charged with 200 ml of sterile maple sap and inoculated with cell suspensions to give the required cell concentration. One hundred ml of the suspensions were transferred to other 500-ml Erlenmeyer flasks and received sufficient formaldehyde to give a final concentration of 5 ppm. The pairs of inoculated flasks (with and without formaldehyde at each inoculum level) were incubated at 7°C or at 27°C without agitation or aeration.

Flasks were sampled immediately after inoculation and every 24 hr for the appropriate time period. The samples, after dilution in sterile distilled water, were plated on tryptose-glucose-beef extract agar (Difco) and incubated at 27°C. Counts were made after 48 hr of growth.

RESULTS

Ps. geniculata was inoculated into sets of the medium to give population levels of 10^3 and 10^4 organisms/ml. These flasks were incubated at 27 or 7°C. Fig. 1 shows the results of the effect of formaldehyde and temperature on the growth of the bacteria. At 7°C, growth in the control flasks (without formaldehyde) was slow, showing an increase only by the second 24-hr period. Maximum growth was not attained even after 72 hr of incubation. In the presence of formaldehyde there was an initial decrease in the number of viable cells. The culture containing, originally, 10^4 cells/ml appeared to recover after 24 hr and the growth rate then paralleled that of the control culture. In the 10^3 cells/ml culture, however, the inhibitory action of the formaldehyde appeared to be greater, and the cell count did not increase appreciably until after 48 hr of incubation.

By contrast with the growth pattern at 7°C, growth at 27°C was almost explosive. Maximum growth was attained in 24 hr or shortly thereafter in both the control and formaldehyde-treated cultures. There is, however, some indication that the formaldehyde may have initially inhibited growth of the cells, since the treated-cell counts at 24 hr are somewhat less than the control counts. Although no viable counts were made before the 24-hr sampling, so the pattern of early growth is not known, it may be that even at this temperature, which favored the rapid growth of the or-

ganisms, the formaldehyde initially affected the cells in a manner similar to its action at 7°C.

Since formaldehyde inhibited the growth of *Pseudomonas* at 7° when a small inoculum was used, the effect on larger inocula was also investigated. Maple sap media, with and without formaldehyde, were inoculated to contain 10^3, 10^4, 10^5, and 10^6 organisms per ml. Growth of these cultures at 7° is shown in Fig. 2. The control cultures originally containing 10^5 and 10^6 organisms/ml grew within 24 hr, indicating that the low temperature had less effect on the continued growth of large numbers of organisms than on cultures containing fewer than 10^4 cells/ml. The presence of 5 ppm formaldehyde, however, was still effective against the higher-inocula cultures, inhibiting growth for at least 24 hr.

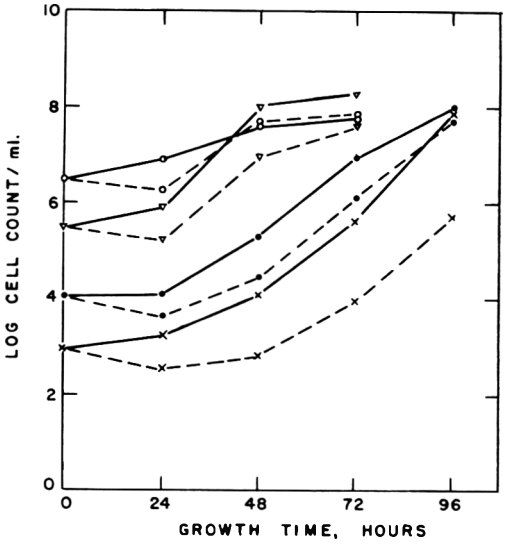

Fig. 2. The effect of inoculum size on the formaldehyde inhibition of the growth of *Ps. geniculata* at 7°C. Control (no formaldehyde), ———; 5 ppm formaldehyde, – – – –.

DISCUSSION

The bacteriostatic, or bactericidal, activity of a compound must be considered in relation to many factors: the organisms involved, the numbers of organism used, concentration of the compound, composition of the medium, and the temperature of incubation. The effect of formaldehyde, an accepted bacterial inhibitor, has been variously assessed, primarily because of variations in the testing conditions. Since this inhibitor has been shown to reduce the degree and rate of contamination of tapholes in maple trees, the effectiveness of low concentrations of formaldehyde on the growth of bacteria in collected sap was evaluated under conditions similar to those likely to prevail in the field.

Ps. geniculata, isolated from sap, was selected as the test organism. Although some yeasts, molds and other bacteria have been found in maple sap this species is the predominating organism. Formaldehyde, at a concentration of 30 ppm, has been shown to be bactericidal to *Ps. geniculata*, but only under laboratory conditions (Costilow *et al.*, 1962). Under field conditions the organisms would be growing in maple sap, which is a minimal medium for maintaining the growth of this organism, and at temperatures of approximately 0°C. In a study of the solution rate of paraformaldehyde pellets designed to be used in the tapholes, the formaldehyde concentration in stationary water approached the 30-ppm lethal dose in 24–48 hr, whereas, in a simulated taphole, flowing water contained about 5 ppm of the inhibitor (Costilow *et al.*, 1962). Under actual conditions, collected sap was found to contain less than 5 ppm formaldehyde in 92% of the samples tested. Thus, from the literature reports, it would appear doubtful that the formaldehyde concentration in the field would be great enough to inhibit the bacteria, although actual trials did show substantial inhibition of taphole contamination. The findings of this limited study indicate that two factors are involved in inhibition of the growth of small populations of bacteria. In the control cultures, low temperatures result in a decrease in the over-all growth rate and there is a definite lag period of about 24 hr before growth occurs.

The inhibitory effect of the formaldehyde can be distinguished from temperature-induced inhibition. At the low inocula levels, particularly at 10^3 cells/ml, the number of viable cells decreases in the first 24 hr, and the amount of growth is appreciably less than in the control cells exposed to low temperature only.

(Low viable counts could possibly be due to a carry-over of formaldehyde to the plating medium. However, cultures were diluted at least 1:100 before plating, thus reducing the maximum amount of formaldehyde possibly carried over to 0.05 ppm. Since 0.5-ml samples of the dilutions were

plated in approximately 10 ml agar medium, the final formaldehyde concentration per plate was less than 0.025 μg.) Even at the higher inocula levels, where the effect of low temperature is not so evident, the cells exposed to formaldehyde undergo an initial period during which no increase in the growth rate occurs. Although the growth rate of the bacteria is very rapid at 27°C, there appears to be some indication that the formaldehyde may have had a transient inhibitory effect readily overcome by the bacteria.

It is not possible to decide from the data whether the action of low temperature and formaldehyde in inhibiting the bacterial growth was additive or synergistic. However, it is suggested that one effect of the lower temperatures in reducing the metabolic activity of the cells is to render them more susceptible to the action of the formaldehyde. This appears to be particularly true at the lower inocula levels.

It is interesting to note that the inhibitory activity of formaldehyde at 5°C is contrary to the findings of McCulloch and Costigan (1936) that lower temperatures decreased the activity of this compound.

Another consideration of this study was the possible effect of the formaldehyde inhibition of bacterial growth on the flavor of maple sirup. Sirup made from sterile sap lacks the full characteristic maple flavor, and some bacterial action (by the proper bacteria) is necessary for complete development of this unknown factor. Reduction of the bacterial population with formaldehyde could result in the production of flavorless sirup. The data indicate, however, that formaldehyde, under the conditions stated, is bacteriostatic, inhibiting growth temporarily. Full development of the flavor factors can be expected to occur in the treated sap, either by outgrowth of the normal flora of the sap or by controlled fermentation with selected bacterial strains.

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The Liquefying Action of Pancreatic, Cereal, Fungal, and Bacterial Alpha-Amylases[a]

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SUMMARY

The starch-liquefying abilities of alpha-amylases from pancreas, cereals, fungi, and bacteria were studied by following changes in the viscosity of potato starch pregelatinized and cooled in the Amylograph under standardized conditions and acted on by the enzyme at 37° C. The optimal pH activity for starch liquefaction was 4.0 for fungal, 4.5 for cereal, 6.5 for bacterial, and 7.0 for pancreatic amylase. A comparison of liquefying action of the enzyme systems at a constant level of dextrinogenic activity showed the pancreatic enzyme most active, followed by cereal, with the fungal least active. The relative differences between the activity of the bacterial and fungal enzymes at their optimum pH's and at 37° depended on the levels of enzyme added. The thermostable bacterial amylase also exhibited high stability toward high acidity. The pattern of starch liquefaction by action of the various enzymes, and the relation between viscosity drop and enzyme activity, are discussed.

Starch is liquefied by an amylase of the alpha type (Kneen, 1950). Through the years, many methods for evaluating starch-liquefying properties have been proposed, but few apparently have the accuracy needed for quantitative determination. Many factors, such as method and temperature of making the paste, and the method and conditions under which the measurements are made, may influence the results. The author has reported on the action of amylases on commercial pregelatinized wheat starch (Pomeranz and Shellenberger, 1962a) and the susceptibility of various starches to the liquefying action of amylases (Pomeranz and Shellenberger, 1962b). These studies were concerned with the action on starches heated at a uniform rate, conditions comparable with those taking place in the bread-baking process. This report deals with a study of the action of each fungal, bacterial, or pancreatic amylase and three cereal (from wheat, barley, and sorghum) amylases on starch pregelatinized under standardized conditions. The Brabender amylograph (Anker and Geddes, 1944), a torsion viscosimeter that provides a continuous automatic record of changes in the viscosity of starch, was used to measure changes in the viscosity of the gelatinized starch, held at 37°C, as a result of the action of known (in terms of dextrinogenic activity) levels of alpha-amylase.

Measurement of alpha-amylase activity through the liquefying action of the enzyme has a number of desirable features. The results are not influenced by the presence of beta-amylase. The viscosity of starch paste is reduced rapidly by the action of relatively small amounts of alpha-amylase. This ability to cause a decrease in viscosity is extremely important in many industrial processes; the high response should be useful in detecting very low levels of alpha-amylase activity.

MATERIALS AND METHODS

Starch. Four samples of unmodified potato starch were obtained from three manufacturers.

Amylase supplements. Four types of amylases were studied: cereal, bacterial, fungal, and pancreatic. Among the cereal amylases, two samples of wheat, two samples of barley, and a sample of

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sorghum amylase were tested. Of the amylases of microbiological origin, 5 fungal and 4 bacterial samples were tested. One of the wheat malt preparations, and also the sorghum malt, were partly purified. The other enzymes were concentrates furnished by three different commercial firms. The dextrinogenic activities of the tested alpha-amylases (expressed as SKB units per gram) were:

Wheat amylases	19 and 414
Barley amylases	52 and 284
Sorghum amylase	840
Fungal amylase	908; 4,590; 5,000; 7,900; and 8,140
Bacterial amylases	5,480; 7,950; 8,000 and 11,880
Pancreatic amylase	1,715

Preparations within each of the classes of an enzyme source had similar properties (optimal pH activity, and liquefying action on a fixed dextrinogenic-activity basis). One preparation of each type of enzyme source was selected for detailed study. Each amylase, except the sample of enzyme of pancreatic origin, was diluted with a 0.2% calcium chloride solution and added to the pregelatinized potato starch in the amylograph bowl. In each case the dilution was prepared in such a way that 1 ml of a 0.2% calcium chloride solution of the enzyme was added. The pancreatic amylase was prepared by suspension of the powder in a 0.02*M* NaCl solution. Similarly, the starch suspension for this enzyme was 0.02*M* NaCl.

Starch dextrinogenic activity of amylases. Dextrinogenic activity was determined by the modified Wohlgemuth method (Wohlgemuth, 1908; Sandstedt *et al.*, 1939; Redfern, 1947) to give the relative alpha-dextrinogenic (SKB) activities of the different enzymes.

Viscosimetric determination of alpha-amylase activity. Unless otherwise stated, 27.5 g of potato starch were suspended in 450 ml of a buffered water solution and placed in the amylograph bowl. The contact thermometer was adjusted to start heating at 30°C, the temperature of the suspension was increased at a constant rate of 1.5°C per min up to 75°C, subsequently cooled at the above rate to 37°C, and kept at 37°C for 10 min. After the enzyme was added, the amylograph was operated at a constant 37°C, and the viscosity was recorded by the instrument during 60 min. Temperature control was automatically provided by the instrument.

Determination of optimum pH. Each of the enzymes was added to a pregelatinized, cooled starch suspension in a 0.02*M* buffer. For each enzymatic system one control determination was made with an unbuffered substrate system, one with an acetate buffer at pH 5.2, and one with a citrate-acetate buffer at pH 5.35. In addition, at least six determinations were run with mixtures of phosphate buffers to cover the tested pH range. For each enzyme system, determinations of optimum pH were made at two enzyme levels: 0.2 SKB and 0.8 SKB units for cereal, pancreatic, and bacterial amylases and 2.0 SKB and 8.0 SKB units for the fungal amylase. These levels of enzyme were chosen so as to obtain the high, as well as the medium, extent of starch liquefaction. The effect of pH on the stability of the enzyme was determined by a prior incubation of 0.8 SKB unit of cereal, bacterial, or pancreatic amylase or 8.0 SKB units of fungal amylase in various 0.2*M* phosphate buffers for 1 hr in the absence of substrate, and subsequently determining the residual alpha-amylase activity after adding them to a pregelatinized and cooled starch suspension readjusted to the optimum pH for the respective enzyme.

RESULTS AND DISCUSSION

The starches from the several manufacturers exhibited pronounced inherent variations in viscosity. Different lots from the same manufacturer gave excellent reproducibility, respectively within 2% and 4%

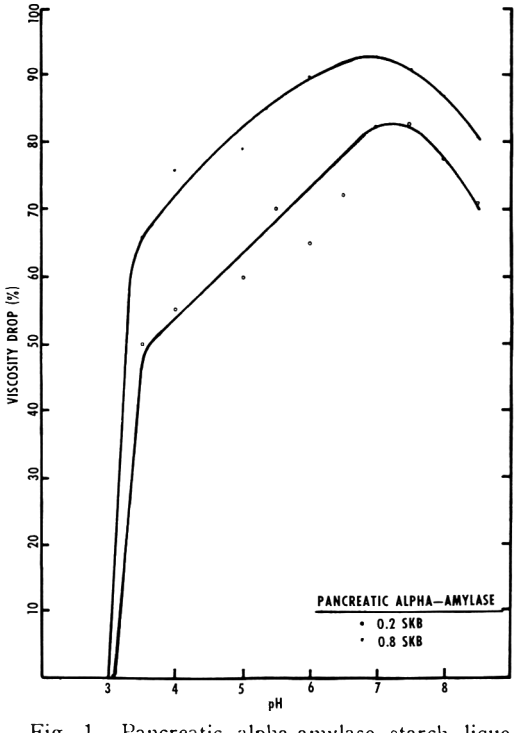

Fig. 1. Pancreatic alpha-amylase starch liquefying activity as influenced by pH (0.02*M* buffer). Conditions: 37°C, 27.5 g potato starch, 0.02*M* sodium chloride, 0.2 and 0.8 SKB units, respectively, 60 minutes' reaction time.

Table 1. Effect of using starches from three manufacturers on determination of wheat α-amylase activity.

	Starch I	Starch II	Starch III
Weight (g)	29.0	32.5	27.5
Reading at end of cooling period (BU)	1000	1000	1000
Drop in viscosity (%) as result of adding			
0.08 SKB unit	54	42	53
0.20 SKB unit	63	55	62
0.80 SKB unit	86	81	85

for high and medium levels of added enzyme. Adjusting the starch concentration to give a reading of 1000 instruments units at the end of the cooling cycle enables one to use potato starches from various manufacturers for determination of alpha-amylase activity (Table 1). The viscosity of a paste of gelatinized starch can be lowered to a large degree by vigorous mechanical action. The change during this process is a disruption of the swollen starch granules, similar to liquefaction under the action of a liquefying amylase. A gelatinized starch paste left without motion tends to exhibit increased viscosity. It is desirable in gelatinization studies to maintain a uniform mixing speed (Holo *et al.*, 1959). To prevent a shearing action due to intensive mixing, the velocity should be regulated so that only the sedimentation of starch grains is prevented. Preliminary results have shown that variations in the speed of bowl rotation influence viscosity measurements. Rotation below 50 rpm was insufficient to prevent settling of the starch; above 100 rpm, mechanical starch disintegration took place. Consequently, all reported determinations were made at 75 rpm.

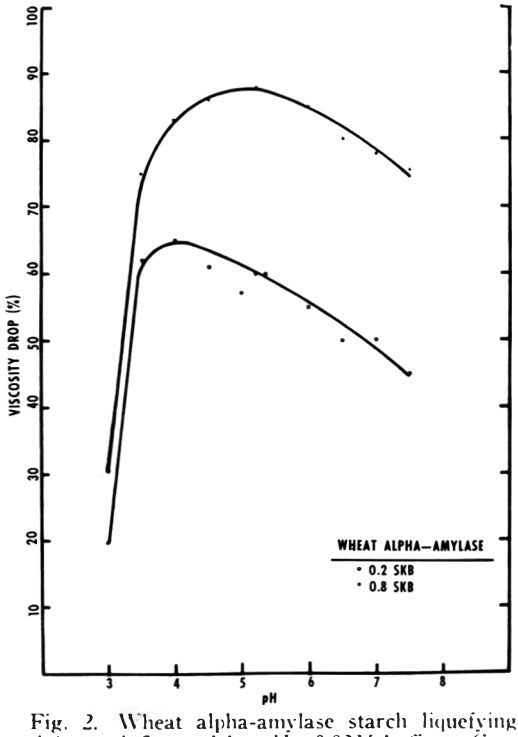

Fig. 2. Wheat alpha-amylase starch liquefying activity as influenced by pH (0.02*M* buffer). Conditions: 37°C, 27.5 g potato starch, 0.2 and 0.8 SKB units, respectively, 60 minutes' reaction time.

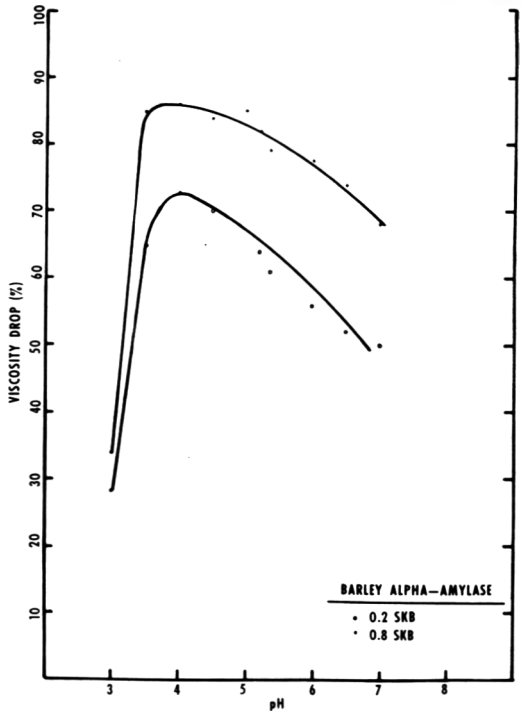

Fig. 3. Barley alpha-amylase starch liquefying activity as influenced by pH (0.02*M* buffer). Conditions: 37°C, 27.5 g potato starch, 0.2 and 0.8 SKB units, respectively, 60 minutes' reaction time.

The activity of amylases tended to increase with increasing temperatures, accompanied by inactivation of most amylases, particularly fungal amylase. Temperatures between 20 and 40°C are chosen most frequently for measurement of amylase activity (Hopkins, 1946). The temperature used in this study

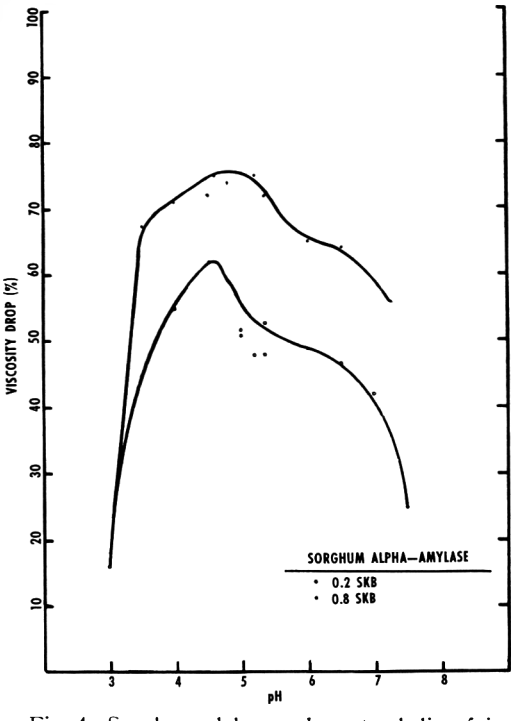

Fig. 4. Sorghum alpha-amylase starch liquefying activity as influenced by pH (0.02*M* buffer). Conditions: 37°C, 27.5 g potato starch, 0.2 and 0.8 SKB units, respectively, 60 minutes' reaction time.

for measurement of alpha-amylase activity was 37°C.

The hydrogen ion activity of the solutions and substrates has been known to have a marked influence on the stabilities and activities of amylases. The optimum hydrogen ion activity for amylases from various sources depends on a number of factors, such as purity of the preparation, enzyme and substrate concentrations, the nature of the electrolytes present, temperature, and the length of time the reactions are carried out. It is therefore not surprising that different optima are found in the literature (Blom *et al.*, 1937; Caldwell and Adams, 1946; Geddes, 1946; Hopkins, 1946; Redfern and Landis, 1946; Sandstedt and Gates, 1954).

In this study neither the presence nor the kind of buffer had, *per se*, any significant effect on amylase activity; only the hydrogen ion concentration affected the results. Figs. 1–6 show the effect of variations in hydrogen ion concentration on starch liquefaction activity of the various alpha-amylases. These results were obtained by plotting the change in viscosity (%) versus the pH of the buffered starch suspension. Optimum pH values for the various systems were at or about: 7.0 for pancreatic, 4.0 for fungal, 6.5 for bacterial, 4.5 for sorghum, 4.5 for wheat, and 4.5 for barley alpha-amylase. For each tested alpha-amylase, using the higher enzyme level resulted in a broader peak of optimum activity than any tested using the medium enzyme level. This was especially pronounced with bacterial amylase.

Table 2. Inactivation of enzymes by holding for 1 hour in 0.2*M* buffer.

Amylase	Level SKB	Viscosity drop (%) after holding at: pH 3.0	pH 5.0	pH 7.0
Sorghum	0.8	0	75	63
Wheat	0.8	0	87	84
Barley	0.8	0	85	86
Fungal	8.0	20	90	89
Bacterial	0.8	46	85	88
Pancreatic	0.8	0	91	93

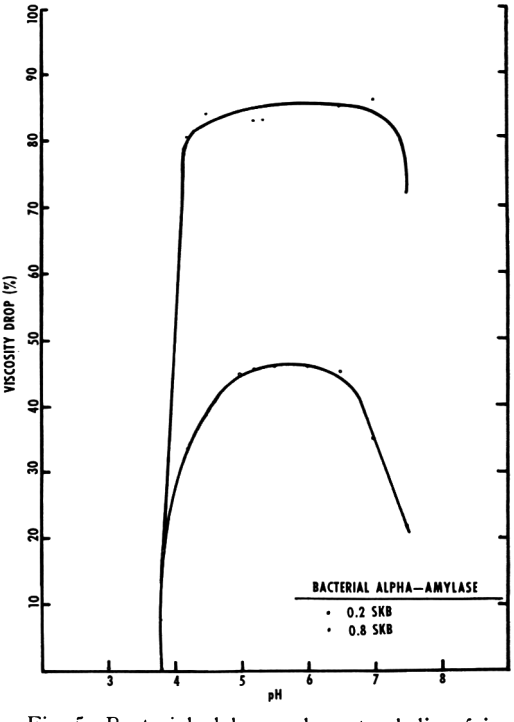

Fig. 5. Bacterial alpha-amylase starch liquefying activity as influenced by pH (0.02*M* buffer). Conditions: 37°C, 27.5 g potato starch, 0.2 and 0.8 SKB units, respectively, 60 minutes' reaction time.

The results shown in Figs. 1–6 and Table 2 seem to point to another feature of bacterial amylases and their high liquefying action. Holding the enzymes in a 0.2*M* buffer of pH 3.0 resulted in complete inactivation of the cereal amylases and 80% inactivation of the fungal amylase. Adding 0.8 SKB unit of bacterial amylase held as acid as pH 2.5 for 1 hour, resulted in a viscosity drop of 27% when the enzyme was allowed to act on a pregelatinized starch suspension buffered at pH 6.5. This stability to acid is especially interesting since the bacterial amylase exhibits a high pH optimum, unlike those of the cereal or fungal amylases.

According to Kneen (1950), the kinetics of the liquefaction of starch by amylases apparently differ somewhat from those operative in starch dextrinization or saccharification. No linear relation could be observed between quantity of enzyme and decrease in viscosity. The viscosity of gelatinized starch drops 50% when only 0.1% of the glucosidic linkages are opened, whereas 7% of the linkages must be broken before the remaining dextrins failed to give a blue color with iodine (Hagberg, 1960). It seems, therefore, that previously reported failures to find linear relation between amylase action and drop in viscosity were due to measurements being made after the action of excessive levels of enzyme and/or for too long times.

This hypothesis is confirmed by findings summarized in Figs. 7–10. In each case, there was a linear relation between enzyme activity and viscosity reduction for the very low levels of enzyme concentration. Higher levels of enzyme resulted in a curvilinear relation. The linear relation was exhibited for the range up to 0.02 SKB for pancreatic alpha-amylase, up to 0.04 SKB for cereal

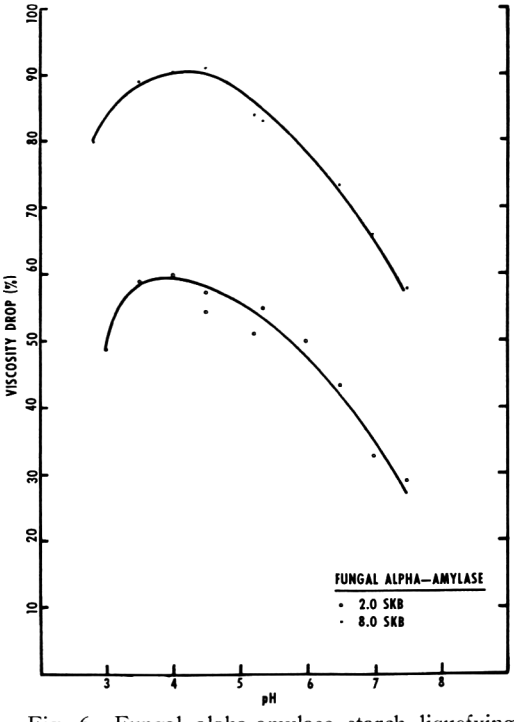

Fig. 6. Fungal alpha-amylase starch liquefying activity as influenced by pH (0.02*M* buffer). Conditions: 37°C, 27.5 g potato starch, 2.0 and 8.0 SKB units, respectively, 60 minutes' reaction time.

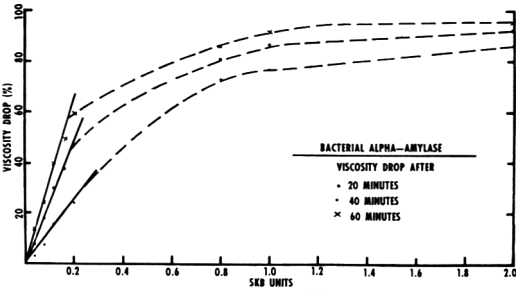

Fig. 7. Pancreatic alpha-amylase starch liquefying activity as influenced by enzyme concentration. Conditions: 37°C, 27.5 g potato starch, 0.02*M* phosphate buffer pH 7.0, and 0.02*M* sodium chloride. Reaction times 20, 40, and 60 minutes, respectively.

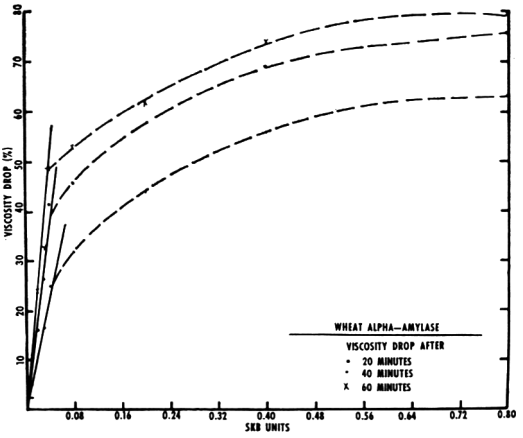

Fig. 8. Wheat alpha-amylase starch liquefying activity as influenced by enzyme concentration. Conditions: 37°C, 27.5 g potato starch, 0.02*M* phosphate buffer pH 4.5. Reaction times 20, 40, and 60 minutes, respectively.

amylase, and up to 0.2 for bacterial or fungal amylase. The above figures refer to SKB units per 27.5 g of starch, with the reduction of viscosity measured after 20 minutes of enzyme action. Comparing viscosity drop after various reaction times showed that, for pancreatic amylase, for example (Fig. 7), there was a linear relation between viscosity drop and enzyme level up to 0.02 SKB unit for the 20-minute reading. The linearity held true up to 0.01 SKB unit for the 40-minute reading, and up to about 0.005 SKB unit for the 60-minute reading. Results were similar for the other amylases. It is possible, however, that, because of prolonged action of very low concentrations of amylase, the results obtained may be affected by denaturation of the enzyme.

Comparing the various amylases on an equal basis of dextrinogenic activity showed that fungal amylase was the least potent in liquefying action, and pancreatic amylase the most potent. Cereal amylases ranked second in potency. Bacterial amylase results were more complex. Over the linear range up to 0.2 SKB unit, bacterial amylase was comparable to fungal amylase, but over the higher range of enzyme activity, at or above 0.8 SKB unit, it resembled cereal amylase. These results point to the possibility of different patterns of liquefying action by amylases from bacterial and fungal sources. Such differences were not noted in comparing the various cereal amylases.

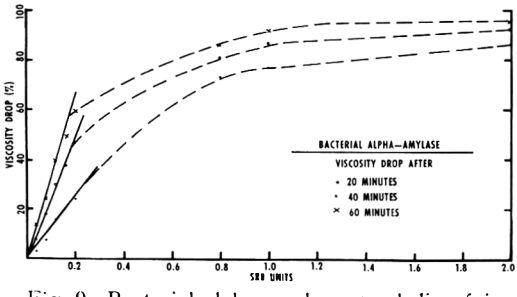

Fig. 9. Bacterial alpha-amylase starch liquefying activity as influenced by enzyme concentration. Conditions: 37°C, 27.5 g potato starch, 0.02*M* phosphate buffer pH 6.5; reaction times 20, 40, and 60 minutes, respectively.

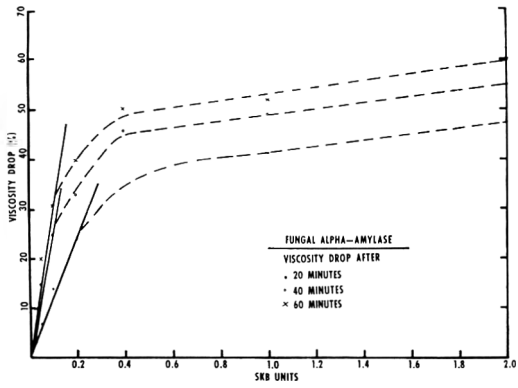

Fig. 10. Fungal alpha-amylase starch liquefying activity as influenced by enzyme concentration. Conditions: 37°C, 27.5 g potato starch, 0.02*M* phosphate buffer pH 4.0; reaction times 20, 40, and 60 minutes, respectively.

ACKNOWLEDGMENTS

The author thanks Dr. P. Nordin, Kansas State University, for the sample of sorghum alpha-amylase; and Morningstar, Paisley Inc., New York, Northern Aroostock Starch Co., Minneapolis, and Stein, Hall and Co., St. Louis, for samples of unmodified potato starch.

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Post-Mortem Changes in the Muscles of Landrace Pigs[a]

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(Manuscript received July 3, 1962)

SUMMARY

A series of chemical and physical measurements were carried out on samples of the longissimus dorsi muscles from 10 Danish Landrace pigs. The samples were taken out immediately after sticking and held at 37°C. Estimations were made at intervals of the content of lactic acid, adenosine triphosphate, creatine phosphate, adenosine nucleotide, and inosine nucleotide, and of pH and extensibility, until rigor mortis was complete. The material could be divided into two groups, A and B. Group A was characterized by a slow fall of pH (max. 0.65 units/hour) and by a slow decrease in extensibility (full rigor at 280 min), whereas group B showed a rapid fall of pH (max. 1.04 units/hour) and a rapid development of rigor (full at 160 min). There was a similarly clear difference between the rates of the other changes. Q_{10} for the rate of pH fall was 2.70 over the temperature range 36—41°C. The day after slaughter, all carcasses that gave samples of type A were of excellent quality, whereas those giving type B were more or less pale and watery. The differences between the two groups could not be explained by different handling of pigs before slaughter.

INTRODUCTION

Many workers have investigated, from diverse points of view, the so-called "muskeldegeneration" (MD) disease of Danish Landrace pigs, which is characterized by a pallid and watery appearance of the meat after slaughter and by more or less severe exudation of sarcoplasmic fluid from it (Ludvigsen, 1954; Wismer-Pedersen, 1959; Lawrie, 1960; etc.) Unfortunately, however, it cannot be said that any satisfactory explanation has yet been given of the basis of the phenomenon. At the same time, it has been established that the disease is on the increase, particularly among the pigs at progeny-testing stations.

From the physiological standpoint, one of the most interesting features of the condition is the occurrence in the meat, ½ to ¾ hr after slaughter, of a higher lactic acid content and a lower pH than normal (Ludvigsen, 1954). This means that, because of the long-drawn-out processes involved in preparation of the carcass for pork or bacon, the meat from such pigs will be exposed to acid conditions (<pH 6.0) while the temperature of the carcass is still high (>35°C), and it is precisely such a combination of high acidity with high temperature that can be shown experimentally to produce in otherwise normal meat the pallor and exudation characteristic of the MD condition (Briskey and Wismer-Pedersen, 1961; Bendall and Wismer-Pedersen, 1962). The problem is, therefore, to explain the exceptionally high rate of lactic acid production and of fall of pH, post-mortem, that is the main factor determining the occurrence of the phenomenon.

Toward this end we undertook a detailed examination of the post-mortem physical and chemical changes occuring during the rigor process in Danish Landrace pigs, which may be compared with work in other countries on animals such as rabbits, horses, whales, oxen, and pigs (Bate-Smith and

[a] The following abbreviations are used throughout: ATP = adenosine triphosphate, LA = lactic acid, CP = creatine phosphate, TSP = total soluble phosphate, AN = adenosine nucleotide, and IN = inosine nucleotide.

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Bendall, 1949; Bendall, 1951; Lawrie, 1953; Marsh, 1952, 1954; Howard and Lawrie, 1956; Lawrie, 1953).

We found, as Lawrie (1960) did with English pigs, that the rate of the changes varies greatly among animals of the same breed, although it is possible to classify them into two broad groups, a slow group and a fast group. It is of some interest that the pH changes in our slow group of Danish Landrace are as fast as, or faster than, the most rapid that Lawrie (1960) described in normal English Large White pigs. Since only the faster of our two groups of pigs showed more or less severe symptoms of wateriness, only this group was physiologically capable of producing a large amount of lactic acid fast enough post-mortem for the carcass to be at a low pH while the temperature was still high. It follows that wateriness is unlikely ever to become a serious problem among the much "slower" English pigs of the present Large White breed.

METHODS

Condition of slaughter. The pigs used in the experiment were all of Danish Landrace with a live weight of 85 to 95 kg, and were purchased from two producers. The pigs were transported to the slaughterhouse, preferably singly in the truck (in only three cases was another pig also present). Slaughter was carried out at the Roskilde Cooperative Slaughterhouse. On only three occasions was there more than one pig on the slaughter floor at once. In nearly all cases the pigs were slaughtered according to the procedure recommended in Denmark; that is, they were shackled by one leg, hoisted, stunned, and stuck. In a few cases the pig was hung up by a shackle round one hindleg for 1 min prior to stunning, but this did not seem to affect the course of post-mortem changes.

Preparation of experimental material. Four min after sticking, a sample of *ca.* 500 g was cut from the longissimus dorsi muscle between the 12th and 15th ribs and placed in a room at a constant temperature of 37°C. The carcass thereafter went through the normal processing procedure.

Three pieces were cut from the main sample of muscle. One piece, *ca.* $5 \times 0.7 \times 0.5$ cm, was used for measurement of extensibility, by the method of alternate loading and unloading described by Bate-Smith and Bendall (1947). Two pieces of *ca.* 20 g were placed on a glass slide, each in its own tube, and were used for pH and chemical determinations. To ensure fully anaerobic conditions, a stream of moist N_2 was passed over the 3 samples.

Measurement of pH and lactic acid content. The pH was measured after maceration of *ca.* 2 g of muscle in 10 ml of 0.005*N* sodium iodoacetate, by means of a Radiometer pH meter 22. To determine the Q_{10} of the rate of pH fall, two pieces were cut in each case from the main samples of muscle from 5 pigs. One piece was placed at 36°C and the other at 41°C, both under moist N_2. Samples for the estimation of pH were taken every 10–15 min.

Lactic acid was measured by the method of Barker and Summerson (1941) except that 4% perchloric acid was used instead of TCA to precipitate the proteins.

Estimation of phosphate, ATP, and nucleotide content. ATP, as labile phosphate, and CP and TSP were measured by the method of Lohmann as modified by Bendall (1951). AD and IN were measured by the spectrophotometric method of Kalckar as modified by Bendall and Davey (1957), using a Zeiss uv spectrophotometer. For measurement of all these values a sample of *ca.* 2 g muscle was weighed out quickly, and macerated in 10 ml of 4% perchloric acid at 3°C, by means of an Ultra-Tarrax mixer. The supernatant, cleared by centrifugation and filtration, was used for the estimations. Samples were taken for the various analyses at intervals of 30 min in the early stages and of 45 min toward the end of rigor.

RESULTS

The results showed that the pigs could be divided, on the basis of post-mortem changes at constant temperature, into two groups, A and B. Group A was characterized by slow post-mortem change, and group B by fast change. Five of the 10 pigs used in the work fell into each group. Figures 1 and 2 show the various changes in the two groups as a function of time. The curves are drawn through the mean values of the various parameters, the vertical lines giving the standard error of the means.

pH changes. The pH values measured 10 min after sticking (pH_{10}) show a scarcely significant difference between the groups, the mean value for group A being 6.78 and for group B 6.66. Because of the much higher rate of pH fall in group B, this small difference becomes completely insignificant when the curves are extrapolated back to zero. In group A the pH falls 0.25 pH unit/hr, until it reaches pH 6.5, when the rate increases. From pH 6.3–5.6 the rate is again nearly constant, but the maximum rate is now 0.65 pH unit/hr. The rate then declines rapidly, and after 270 min

there is no further change in pH. In group B, however, the rate is high from the start, and 70 min after death (at pH 6.0) reaches a maximum of 1.05 units/hr. The pH fall is complete in this group after 150 min. The pH at completion of rigor (ultimate pH) has a mean value of 5.40 in both groups.

Temperature coefficient (Q_{10}) of the pH changes. When the rates of pH fall in samples of longissimus dorsi muscle from the same pig are compared at 36 and 41°C, it is found that the average Q_{10} in this temperature range is 2.70 with a standard deviation of $\pm$ 0.15 (5 estimations). There is no significant difference between the Q_{10} values for the fast and slow portions of the pH/time curves, nor does the Q_{10} depend on the absolute rates of change, whether of type A or B. The average apparent energy of activation of the processes underlying the pH change, calculated from the Hood-Arrhenius equation, is $\sim$ 19 kcal per g mole lactic acid from 36 to 41°C.

Lactic acid formation. The formation of lactic acid in the two groups agrees more or less exactly with the corresponding pH values. At 10 min after death there is a higher content in group B ($\sim$ 51 μmol/g) than in group A (38 μmol/g), whereas the final value in both groups is $\sim$ 130 μmol/g in spite of the fact that the rate of formation is much greater in group B. Correlation of the pH with the lactic acid values gives $\frac{\Delta LA}{\Delta pH} = 56$ μmol/pH/g for both groups.

Changes in ATP (7 min P). The rate of disappearance of ATP differs markedly in the two groups. In group A, the ATP concentration remains high and nearly constant for the first 30 min, and then falls at constant speed until 280 min, whereas in group B it falls rapidly from the start until 150 min. When the ATP/time curves are extrapolated back to zero time, the ATP level at the moment of death appears to lie between 5.7 and 7.5 μmols/g. The average values of the two groups do not differ significantly from one another at this time (7.1 $\pm$ 0.2 μmol/g (s.e.) for A, and 6.8 $\pm$ 0.4 μmol/g for B). Because of these random variations in initial level, the ATP concentrations are given on the curves in Fig. 1 as percent of the values at zero time.

The above values for ATP were calculated from 7-min P values, but, as Bendall and Davey (1957) have shown, they can also be calculated from the increase in IN. Curves drawn from the IN estimations show initially good correlation with the above values, but toward the end of rigor they

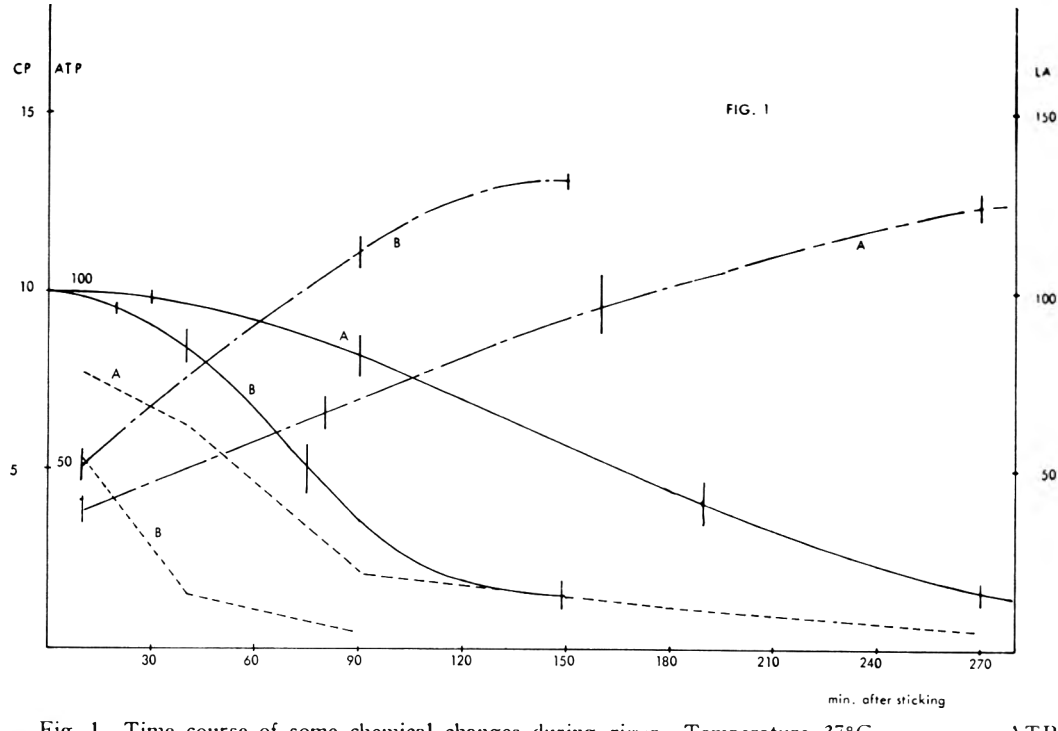

FIG. 1

Fig. 1. Time course of some chemical changes during rigor. Temperature 37°C ———— ATP as % of zero-time value. — — — LA μmol/g. — — — — — CP μmol/g. A indicates curve for group A pigs, mean of 5 pigs. B indicates curve for group B pigs, mean of 5 pigs. Vertical line gives the standard error of the mean.

give a lower, and probably more reliable, measure of the ATP level. This is because the 7-min P values taken at the end of rigor include some phosphorous from the hexose mono- and di-phosphates formed during the process.

The total turnover of labile phosphate can be calculated from the known values of CP, IN, and pH or lactic acid (Bendall and Davey, 1957), and is found to reach a maximum in group A of 1.04 μmols P/min/g at pH 6.0, and in group B of 1.63 μmols P/min/g at pH 5.95. These two rates differ significantly from one another at $p < 0.2$.

Creatine phosphate. The CP concentration at 10 min after death varies from 3 to 11 μmols CP/g. The average value for group A is 8, and for group B 5.5 μmols/g. The CP content falls very rapidly in group B and is <2 μmol/g after 35 min, whereas this value is reached in group A only after 100 min. The TSP content is nearly identical in both groups (1.85 mg P/g in A, and 1.82 in B).

Extension changes. The extension of the muscle decreases in a different way in the two groups. Group A is characterized by a drop of about 10%, which occurs comparatively quickly, but after this the extension decreases slowly until rigor is complete at *ca.* 280 min. In group B, the extension falls initially at the same rate as in group A, but the ensuing slow phase lasts only a short time, and after 80 min the extension falls precipitously, at a maximal rate three times as great as that in group A, so that rigor is complete at *ca.* 160 min. When the extensibility changes are correlated with the ATP concentration, it is found that the rapid decrease in extensibility first sets in when the ATP concentration is *ca.* 30% of the initial value, in both groups.

Assessment of meat quality. The day after slaughter, samples from the carcasses were evaluated for quality. All the samples from group A were of satisfactory quality, but those from group B were more or less of pale and watery appearance.

Effect of ante-mortem treatment. It was attempted to relate the ante-mortem treatment to the course of the post-mortem changes, and especially to see whether short-term excitement and exertion would provoke a rapid fall of pH.

Three pigs were therefore hung up for one minute by a hind-leg and then stuck. This did not appear to have any effect on the subsequent rate of pH fall. Similarly, it did not seem that the violence of the convulsions after sticking were of any special significance.

DISCUSSION

Our investigations differ essentially from earlier work by Briskey and Wismer-Pedersen (1961) on the muscles of Landrace pigs in that they were carried out at constant temperature. Under these conditions our material can be divided, with respect to post-mortem processes, into two groups, A and B, which differ from one another in the time for completion of the changes. Thus, the changes are complete 120 min sooner in group B than in group A. At first sight, this difference might be attributed to the lower initial pH and CP content in group B, but this can be only part of the explanation, because there is also a highly significant difference between the rates of change in the two groups. It was not possible from the experiments to explain the reason for these differences in rate, because all attempts failed to provoke faster changes by varying the pre-mortem handling of the animals.

Experiments of the same kind as those carried out here have been reported for many different animals: rabbit (Bate-Smith and Bendall, 1947, 1949; Bendall, 1951), whale (Marsh, 1952), ox (Howard and Lawrie, 1956), horse (Lawrie, 1953), and pig (Lawrie, 1960). Apart from the last author and Marsh (1952), no one has previously found that the processes in the same muscle from different animals of the same breed treated in the same way could be classified into two distinct groups according to the rates of the reactions.

Lawrie (1960) undertook a long series of investigations on English pigs, mostly of the breed of Large White × Middle White. His results differ markedly in many respects from those found here, although they most nearly resemble those described for group A. A time course similar to that in our group B is not mentioned. Lawrie finds a typical pH of *ca.* 6.70 at 60 min post-mortem (pH_{60}) and an ultimate pH of *ca.* 5.5, which agree with those for our group A (6.63 and 5.40, respectively). On the other hand, there is a big difference in the rate of pH fall; thus, the pH in Lawrie's experiments was 6.30 at 160 min and 5.80 at 260 min, whereas the corresponding values for group A at these times were 5.8 and 5.42. Lawrie also finds a very large variation in rate, so that the time required for the pH to fall from 6.70 to 5.50 can vary between 180 and 460 min, compared to an average of 170 min for group A.

Even from his average curve, however, the maximum rate of pH fall, at pH 6.0, is only ~0.33 units per hour, compared to 0.65 and 1.05 unit/hr, respectively, in our groups A and B, and with 0.65 units per hour in the rabbit psoas and beef longissimus dorsi muscles (Marsh, 1954). It thus appears that English pigs of the Large White × Middle White breed are remarkable for an exceptionally slow fall of pH at 37°C.

The Q_{10} of the reactions underlying the fall of pH has been studied in detail by Marsh (1954) in beef longissimus dorsi. He found the Q_{10} to increase very rapidly at temperatures above 30°C, where it is ~2.0, to ~3.7 at 36°C and to ~6.8 at 40°C. Our results with Landrace pigs show a Q_{10} of ~2.70 between 36 and 41°C, compared with Marsh's value for beef of ~5.2, the apparent energy of activation in the two cases being ~19 and ~32 kcal per g mole lactic acid, respectively. Compared with these high values at 38.5°C, however, the energy of activation at room temperature is less than 4 kcal per g mole. Thus, the greatest care must be taken in interpreting rates of pH fall, particularly in the intact carcass, where measurements of temperature must be very accurate before it can be decided whether an apparently high rate of pH fall is due to a real change in the absolute rates or merely to a higher average temperature.

Ludvigsen (1955) proposed that the different rates of pH fall might be explained by differing buffer capacities. Lawrie measured the buffering capacity in the longissimus dorsi of normal and MD pigs, and found, respectively, 59.1 and 56.6 μeq H^+/pH/g. We measured the buffering capacity in terms of $\Delta LA/\Delta pH$ and found 56 μequiv H^+/pH/g for both groups of pigs. We have also estimated the buffering-capacity by titrating 5 g of muscle macerated with 20 ml of 0.1*M* KCl + 1 ml of 0.1*M* iodoacetate. We titrated the samples with 0.1*N* NaOH in intervals of *ca.* 0.10 pH units from pH 5.4 to 7.2. No difference could be established between normal and MD pigs. The buffer capacity, in μeq H^+/pH/g, was not constant over the range, but varied as

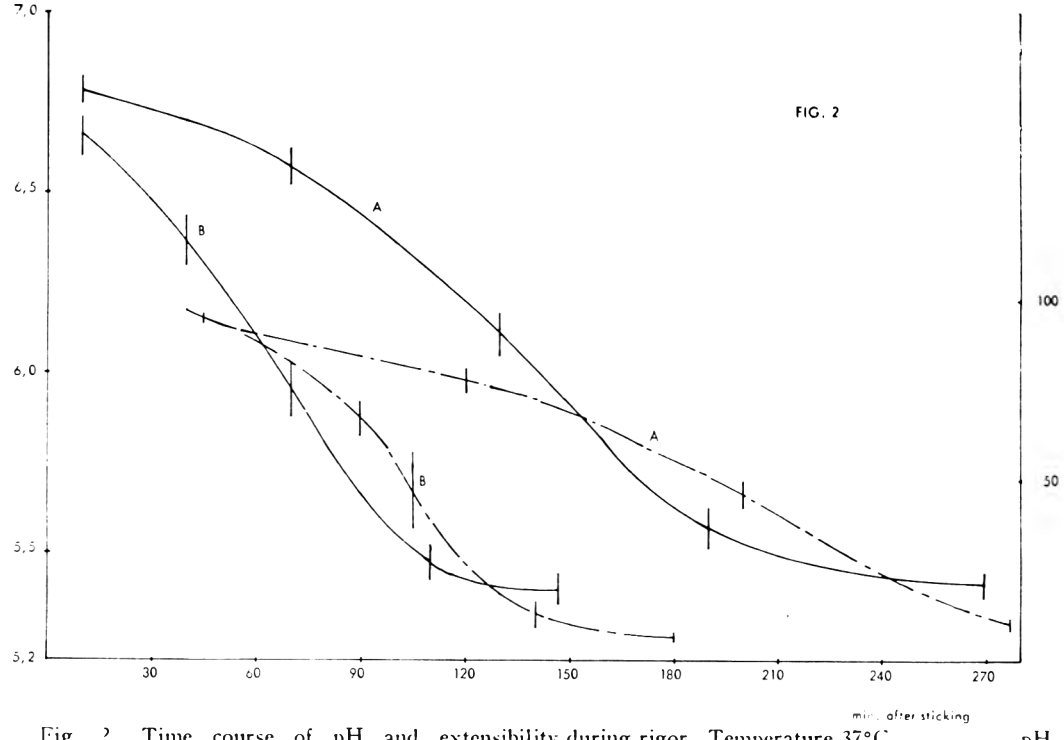

Fig. 2. Time course of pH and extensibility during rigor. Temperature 37°C. ———— pH. — — — — extensibility as % of initial length. A indicates curve for group A pigs, mean of 5 pigs. B indicates curve for group B pigs, mean of 5 pigs.

follows: 57 at pH 5.60, increasing evenly to 65 at pH 6.80, and then falling again to 56 at pH 7.20.

The curve given by Lawrie (1960) showing ATP and CP against time, is in good agreement with our group A, although the ATP concentration in his experiments was a little higher than ours (*ca.* 8 μmol/g at 60 min, against *ca.* 6.7 μmol/g). These differences decrease with time. On the other hand, Lawrie's results on the extension changes differ very greatly from ours. He found, for instance, that the extension declines extremely rapidly after 120 min and that the change is complete at 180 min; the concentration of ATP and the pH are therefore very high, both at the beginning and end of the rapid phase of rigor. From Lawrie's Figs. 2 and 4, the pH is 6.5 and the ATP *ca.* 6.5 μmols/g at the beginning of rigor, and 6.1 units and 3.5 μmols/g at the end. In our group A, the fall of extension is stretched out over a longer interval, whereas the fall of pH is quicker. Indeed, the rapid fall of extension begins in both groups, A and B, only when the pH has reached 6.0 and the ATP concentration has fallen to *ca.* 30% of the initial value (i.e., *ca.* 2.1 μmols/g). Moreover, the fall in extension reaches completion in both groups at the same time as the fall of the pH and of ATP. Considerably better agreement is obtained, however, by comparing our results for the relationship between pH, ATP, and extension with those reported for rabbits, ox, and horse.

We have calculated the maximal rate of turnover of ATP from our results and found it to be 1.04 μmol P/min/g for group A, and 1.63 μmol P/min/g for group B. Bendall (1951) gives a value of 0.74 μmol P/min/g for rabbit, and, calculating from Lawrie's results (1953 and 1960), we find a value of 0.78 for horse and 0.55 μmols/min/g for English pigs. Thus, the rate for our group A pigs is of the same order of magnitude as those for rabbit and horse. The rate in English pigs is, however, very low, in good agreement with the slow fall of pH, whereas that for our group B (1.63) is seen to be considerably higher than any reported for other species of animal.

Briskey and Wismer-Pedersen (1961) had earlier investigated the various post-mortem changes in pigs of Danish Landrace, but it is difficult to compare their results with ours, because they cooled the carcasses from which they took the samples, so that the temperature was varying throughout the experiments. On the basis of their results they divided the pigs into 4 classes according to the rate and course of pH fall after death. The changes in their groups 1, 2, and 3 do not, however, show anything that would not correspond to our group A, if the temperature had been constant at 37°C. Group 4, on the other hand, is characterized at 1½ hr after death by a pH of 5.1–5.4 in the carcass, as measured by a probe glass electrode, and this would seem to indicate a considerably faster fall of pH than that found in our group B. It must he remembered, however, that the temperature during the first 90 min after sticking is probably higher than 37°C, and this must necessarily mean an increased rate of chemical reaction, as shown by the high Q_{10} of 2.70 found in this temperature range. In addition, the pH values as measured by the probe electrode at this higher temperature will be lower by *ca.* 0.2 units than ours, which were measured at room temperature. This is explained by the fact that the pK values of the buffer systems of the meat are highly temperature-dependent (Bendall and Wismer-Pedersen, 1962). This means that muscles from group 4 animals at 90 min after sticking would give pH values of 5.3 to 5.6, if measured after maceration by our method. The pH 90 min after death in our group B was 5.65. There is, therefore, nothing to suggest that the pigs of Briskey and Wismer-Pedersen's group 4 could not be included in group B.

Lastly, it should be noted that we always observed in our experiments that meat which was allowed to go into rigor at a constant temperature of 37°C became watery and of pallid appearance, similar to the typical MD condition. Bendall and Wismer-Pedersen (1962) also observed that the water-retention, extractability and titration curves of meat going into rigor at 37°C closely resemble those of MD pigs. It is, therefore, probable that the reason for the pale and watery

appearance of the meat is the combination of high temperature and low pH, and that this could be prevented from occurring, even in pigs of type B, if the carcass could be cooled fast enough to below 30°C.

CONCLUSION

It is shown from the experiments that, with reference to the post-mortem changes, pigs can be divided into two groups, a slow (A) and a fast (B). Nothing similar has been observed earlier. Pigs belonging to group A were all of good quality, whereas group B pigs were all more or less of MD type. It thus appears that, 4 minutes after sticking, it is already certain whether MD (watery and pallid meat) will or will not develop.

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Changes in Carbohydrate and Phosphorus Content of Potato Tubers during Storage in Nitrogen

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SUMMARY

When potatoes are stored in nitrogen at low temperatures, starch decomposition, release of starch-bound phosphorus, and accumulation of inorganic phosphorus are less than during storage in air. Although there is a complete suppression of sugar accumulation, evidence is presented that the starch removed appears as substances, probably end-products of anaerobic glycolysis, that are volatile at the temperatures used to determine percent dry weight. The decreases in starch-bound phosphorus are balanced by a corresponding increase in inorganic phosphorus in both air-stored and nitrogen-stored potatoes. The decrease in the phytic acid phosphorus in air-stored tubers can be accounted for largely as an increase in the fraction containing phosphoproteins, nucleic acids, and phospholipids, whereas the corresponding loss of phytic acid phosphorus in nitrogen-stored tubers appears largely in the fraction containing nucleotide coenzymes and hexose phosphates. The significance of these findings is discussed in terms of the requirements for high-energy phosphate derived from the respiratory process and needed for biosynthesis.

Under stress conditions such as exposure to temperature extremes, desiccation, or ionizing radiation, potato tubers develop sugars at the expense of starch (Schwimmer and Burr, 1959). This sugar accumulation requires energy, as evidenced by previous observations that sucrose does not accumulate in potatoes stored anaerobically (McCready, 1948; Nelson and Auchincloss, 1933) or in the presence of respiration inhibitors (Craft, 1956), and that it disappears in anaerobically stored potatoes previously stored at low temperature in air (Barker and el Saifi, 1952). This suppression of starch-to-sucrose conversion may be due to by-passing of the normal synthetic pathway for sucrose synthesis, via glycolysis, or there may be a suppression of starch decomposition as well. Evidence in this paper indicates that both processes are operable in potatoes stored at low temperatures in nitrogen.

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MATERIALS AND METHODS

Potatoes of mature White Rose and Kennebec varieties used in previous studies (Samotus and Schwimmer, 1962a) were stored for 5 weeks after harvesting. One-kilogram lots from each variety were then transferred to desiccators containing nitrogen gas and alkaline pyrogallol (500 g KOH and 150 g pyrogallol in 1 L). The desiccators were thoroughly flushed by repeatedly reducing the pressure within the desiccators and re-introducing nitrogen gas. They were then placed at 32°F for 6 weeks. Every 5 days during this storage period, the above flushing procedure was repeated. Other 1-kg lots of each variety were also placed at 32°F exposed to air.

Details of the methods of fractionation of the tubers and analysis are given in previous publications (Samotus and Schwimmer, 1962a,b,c). Solids and carbohydrate contents are expressed as percent of fresh weight before storage at 32°F, and the phosphorus components as mg of phosphorus per 100 g of fresh tubers before storage.

RESULTS

Changes in carbohydrate composition (Table 1). Total weight losses were considerable in the storage period (at 25°) between harvest and storage

Table 1. Weight and carbohydrate changes (% of fresh weight) in potatoes stored in air or nitrogen at zero degrees.

Component or measurement	Variety [a]	Before 0° storage (B)	After 0° storage in: Air (A)	After 0° storage in: Nitrogen (N)	Differences [b] A-B	Differences [b] N-B
Tuber wt loss	W	4.85 [c]	6.45 [d]	5.48 [d]	+1.90	0.0
	K	7.01 [c]	9.21 [d]	6.39 [d]		
Dry weight [e]	W	23.2	22.6	20.7	−0.2	−1.8
	K	22.8	23.0	21.7		
Starch	W	18.5	14.3	15.5	−3.8	−2.3
	K	18.3	14.9	16.8		
Non-starch solids	W	4.7	8.3	5.2	+3.6	+0.4
	K	4.5	8.1	4.9		
Total sugars	W	0.58	4.44	0.55	+3.5	−0.1
	K	0.44	3.49	0.35		
Sucrose	W	0.38	2.47	0.31	+2.0	−0.1
	K	0.32	2.31	0.24		
Fructose	W	0.08	1.38	0.15	+1.2	+0.1
	K	0.05	1.06	0.10		
Glucose	W	0.12	0.59	0.09	+0.3	0.0
	K	0.07	0.12	0.00		

[a] W = White Rose variety; K = Kennebec variety.
[b] Difference expressed as mean of difference of both varieties.
[c] Loss of weight in the time interval after harvest and before storage at 0°.
[d] Loss of weight in the time interval after harvest and after storage at 0°.
[e] As measured by weighing the samples before and after heating 1 hr at 30°.

at 32°F. The subsequent storage at 32°F in nitrogen did not result in appreciable weight loss. However, significant losses in "dry weight" (as measured by weighing the samples before and after heating 1 hr at 130°) (Samotus and Schwimmer, 1962a) did occur in tubers stored at 32°F in nitrogen, but not in air. The starch contents of both air- and nitrogen-stored samples decreased, with the air-stored tubers showing the greater loss. In contrast, there was no change in the already very small sugar content of nitrogen-stored tubers, whereas the sugar content of the air-stored samples increased, as expected, to about the same extent that the starch content decreased. The changes in the levels of non-starch solids show about the same pattern as those of the sugars, although the initial content of the former was about 10-fold that of the latter. The increase in sugars stored in air was due mainly to the appearance of sucrose and, to a lesser degree, fructose. Only the White Rose variety accumulated glucose to any appreciable extent.

Changes in phosphorus composition (Table 2). The results obtained from storage in air are in agreement with those previously reported (Samotus and Schwimmer, 1962a,b,c). In air at 32°F the starch-bound phosphorus decreased both with respect to unit weight of the tuber (P_{st}) and per unit weight of starch (P_{ss}), as did the trichloroacetic acid (TCA)-soluble organic phosphorus (P_o); whereas inorganic phosphorus (P_i) and TCA-insoluble non-starch phosphorus (P_r) increased. The change in P_o was due almost entirely to decrease in phytic acid phosphorus (P phytic) whereas the level of TCA-soluble, non-phytic acid phosphorus (P ester) remained constant.

After storage in nitrogen, at 32°F, the level of starch-bound phosphorus (P_{st}, P_{ss}) decreased, and that of inorganic phosphorus (P_i) increased as in the case of air-stored potatoes. However, as in the case of starch levels, the change was not as great as in air. In contrast to the air-stored potatoes, the P_o and P_r levels remained constant. However, the invariance of P_o was due to the balancing of an increase in phytic acid phosphorus (P phytic) by a decrease in the non-phytic acid fraction of the TCA-soluble organic phosphorus (P ester). Finally, it may be pointed out that the increase in starch-bound phosphorus per unit weight of starch (P_{ss}) upon storage in air or nitrogen confirms previous calculations that the

amount the starch lost upon storage is richer in phosphorus than either the starch remaining or the starch present before storage. This indicates that the process of starch decomposition proceeds via similar, if not identical, mechanisms in air and in nitrogen, and that the phosphorus is distributed inhomogeneously within the starch granule (Samotus and Schwimmer, 1962c).

DISCUSSION

The small loss of total weight of nitrogen-stored tubers can be reconciled with the relatively large decrease in percentage dry weight only on the assumption that certain substances other than water are volatilized during the determination of percent dry weight. On the basis of this assumption, the decrease in starch content of the nitrogen-stored tubers may be accounted for largely as an increase in volatile substances, whereas the corresponding decrease in starch content of air-stored tubers appears as increase in sugar content (Table 3). Thus, anaerobiosis does not completely suppress the decomposition of starch, indicating that this process, in contrast to the accumulation of sugars, is not entirely an energy-requiring process. These volatile substances are likely to be the products of anaerobic glycolysis. Thus, Barker and Saifi report the accumulation of lactic acid, ethanol, and acetaldehyde during the prolonged storage of tubers in nitrogen (Baker and el Saifi, 1952). The requirement for oxygen for the synthesis of sucrose is consonant with the need to regenerate uridine diphosphate glucose for the progressive accumulation of this sugar (Schwimmer and Rorem, 1960).

Table 2. Changes in phosphorus (mg/g fresh weight) in potatoes in air or nitrogen at zero degrees.

Fraction or measurement [a]	Variety [b]	Before 0° storage (B)	After 0° storage in:		Differences [c]	
			Air (A)	Nitrogen (N)	A-B	N-B
P_{st}	W	16.5	11.6	13.2	−4.8	−2.7
	K	19.7	14.9	17.5		
P_i	W	15.7	19.6	18.3	+3.8	+2.6
	K	19.4	23.2	22.0		
P_o	W	18.9	17.6	18.8	−2.2	−0.3
	K	20.3	17.2	19.7		
P_s	W	11.7	12.8	12.0	+1.7	0.0
	K	14.6	16.9	14.2		
P phytic	W	14.9	13.3	12.6	−2.5	−2.5
	K	17.0	13.7	14.4		
P ester	W	4.0	4.3	6.2	+0.3	+2.0
	K	3.3	3.5	5.3		
P_{st}	W	89.3	81.1	85.4	−8.0	−3.5
	K	107.8	100.0	104.3		
F_p	W	161.0	178.0	168.0	124.0[d]	131.0[d]
	K	134.0	146.0	140.0	102.0[d]	98.0[d]

[a] P_{st} = mg of starch-bound phosphorus per 100 g of fresh tubers.
P_i = Inorganic phosphorus.
P_o = TCA-soluble organic phosphorus.
P_s = TCA-insoluble non-starch phosphorus.
P phytic = phytic acid phosphorus.
P ester = TCA-soluble non-phytic acid phosphorus.
P_{st} = mg of starch-bound phosphorus per 100 g of starch.
F_p = Periodicity of occurrence of phosphorus in amylopectin moiety of starch expressed as number of glucosyl residues per atom of amylopectin-bound phosphorus.
[b] W = White Rose variety, K = Kennebec variety.
[c] See footnote 2, Table 1.
[d] F_p of amylopectin moiety of starch lost during storage.

Table 3. Distribution of components as percentage of non-moisture constituents.[a]

Component	Before storage (B)	After storage in:		Differences	
		Air (A)	Nitrogen (N)	A-B	N-B
Starch	80	64	70	−16	−10
Sugars	2	18	2	+16	0
Other solids	18	18	20	0	+2
Volatiles[b]	0	0	8	0	+8

[a] Average value of Kennebec and White Rose varieties.
[b] Volatiles other than water.

Table 4 expresses the phosphorus distribution as a percentage of the total phosphorus content; values for both varieties were averaged. This table clearly shows that all of the starch-bound phosphorus that disappeared during storage in either air or nitrogen could be accounted for as inorganic phosphorus. The decrease in the phytic acid phosphorus content of the air-stored tubers corresponds to an increase in the TCA-insoluble non-starch phosphorus, consisting largely of phosphoproteins, nucleic acids, and phospholipids. The formation of ester bonds in these compounds apparently requires a source of high-energy phosphate. The fraction of non-phytic acid phosphorus that contains nucleotide coenzymes and hexose phosphates, did not increase during cold storage in air. Mori *et al.* (1960) showed that the extent of incorporation of tracer inorganic phosphates into these classes of compounds increases in potatoes stored in the cold. In nitrogen-stored tubers, there appears to be a net synthesis of these phosphate esters apparently at the expense of phytic acid phosphorus. The pattern of change suggests that the phosphorus of phytic acid, which under aerobic conditions is destined to appear as a net increase in the fraction containing phospholipid, nucleic acids, and phosphoprotein, is prevented from being thus converted under anaerobic conditions, because there is no supply of high-energy phosphate derived from the respiratory processes. The accumulation of this pool of active metabolic phosphorylated intermediates may also be related to the partial suppression of net starch decomposition.

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Table 4. Distribution of phosphorus as percentage of total phosphorus.[a]

Phosphorus fraction	Before storage	After storage in:		Differences	
		Air (A)	Nitrogen (N)	A-B	N-B
Starch	26	20	22	−6	−4
Inorganic	26	32	30	+6	+4
Phytic Acid	23	19	19	−4	−4
TCA-insoluble	19	23	20	+4	+1
TCA-soluble[b]	6	6	9	0	+3

[a] Average values of both varieties.
[b] Not including phytic acid.

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Factors Affecting Enzymatic Solubilization of Beef Proteins

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SUMMARY

Investigation was made of the effect of pH, enzyme concentration, cysteine, sodium chloride, and temperature on the extent of solubilization of 0.10*N* sodium hydroxide-soluble proteins, elastin, and collagen of biceps femoris muscle of beef by ficin and bromelain. All protein fractions of beef are solubilized maximally at pH 7 and 80°C by both ficin and bromelain. Ficin is particularly effective in the solubilization of elastin, even at 20°C. Cysteine and sodium chloride increase the extent of solubilization, especially of collagen and elastin (sodium chloride only). The available data indicate that collagen must be denatured before ficin and bromelain can solubilize it.

Previous workers have shown that a number of proteolytic enzymes can solubilize the proteins of beef muscle and that there is a certain degree of selectivity among these enzymes in solubilizing the different protein fractions (Gottschall and Kies, 1942; Tappel *et al.*, 1956; Wang *et al.*, 1958). Ficin, bromelain, and papain, all from tropical plants, and trypsin, from the pancreas, possess collagenase and elastase activities. But the microbial enzymes Hydralase TP, Hydralase D, fungal amylase, protease 15, and HT proteolytic had little or no effect on collagen. [The source of these microbial enzymes is given by Wang *et al.* (1958)].

While it is clear that many of the proteolytic enzymes can solubilize the proteins of beef, it is not clear how such factors as enzyme concentration, pH, temperature, ionic strength, activators, and native vs. denatured protein affect the results. Obviously, all of these are important if the enzymes are to be properly used.

The present work was carried out to evaluate the influence of these factors on the solubilization of beef proteins by ficin and bromelain.

MATERIALS AND METHODS

Materials. Ficin was obtained from Merck and Co., and bromelain from Dole Corporation. Ficin contained 4.94% water and 79.1% protein on a moisture-free basis; bromelain contained 3.20% water and 51.5% protein on a moisture-free basis. Protein was determined by the biuret method (Layne, 1957). Casein was of "Hammersten" quality, obtained from Nutritional Biochemicals Corp. The ground-meat substrate was prepared by first freeze-drying the biceps femoris muscle of beef in a Stokes 2004L lyophilizer at a pressure of 0.1 mm Hg and a plate temperature of 27°C. It was then passed through a Wiley Mill equipped with a 10-mesh screen and stored at −27°C. The moisture content was 4.24% as determined in a vacuum oven. Cysteine was a reagent-grade product of Nutritional Biochemicals Corp. All other salts used were of reagent grade.

Methods. Bromelain and ficin were assayed on casein by a slight modification of the method of Kunitz (1947). The reaction mixture contained 1% casein, $1.25\times10^{-2}M$ each of cysteine and versene, and 0.2*M* phosphate buffer, pH 7.0. At 35°C, ficin had 1.05 units of activity per mg of enzyme, and bromelain had 0.721 units of activity per mg enzyme against casein. Under the same conditions, but without cysteine and versene added, ficin and bromelain respectively had 0.21 and 0.496 units of activity per mg of enzyme.

Separation of beef proteins into soluble protein, non-protein, collagen, and elastin fractions by sodium hydroxide extraction, trichloroacetic acid precipitation, and hot-water extraction was carried out essentially as described by Miyada and Tappel (1956), who modified a method first used by Lowry *et al.* (1941). Triplicate 1-g samples of ground, freeze-dried biceps femoris muscle of beef were rehydrated with 5 ml of water or appropriate buffer or salt solution for 30 min at room temperature. Enzyme and water were added to bring the total volume to 10 ml. Incubation

was then carried out under the appropriate conditions. Reaction was stopped by addition of 20 ml of 0.10*N* sodium hydroxide. Subsequent extractions and precipitations were carried out as described by Miyada and Tappel (1956), using one-fifth the volumes they used. Nitrogen content of each fraction was determined by micro-Kjeldahl. All values, the average of at least three determinations, are reported as percent of total nitrogen. The data in Figs. 1–5 represent a decrease in soluble proteins nitrogen, elastin nitrogen, and collagen nitrogen, and an increase in non-protein nitrogen, over the control. It should be noted that the data do not express the results in percentage peptide bonds broken, but rather reflect changes in the solubility properties of the protein fractions.

RESULTS AND DISCUSSION

Effect of pH. Fig. 1 shows the effect of pH on the disappearance of beef proteins and the appearance of non-protein nitrogen when ground meat is treated with ficin and bromelain at 60°C for 1 hour. Maximum activity of both these enzymes is near pH 7 for solubilization of all fractions of beef proteins. It is near pH 7 for casein and gelatin hydrolysis by ficin (Whitaker, 1957a,b). In contrast to the results reported here for elastin in freeze-dried beef, purified elastin has been found to be hydrolyzed more rapidly at pH 5.0–5.5 than at pH 7 (Yatco-Manzo and Whitaker, 1962). It was previously shown that the maximum activity of

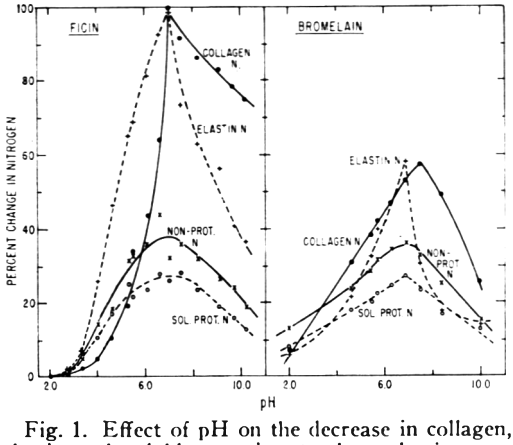

Fig. 1. Effect of pH on the decrease in collagen, elastin and soluble proteins (and on the increase in non-protein nitrogen) in beef treated with ficin and bromelain at 60°C for 60 min. Enzyme concentrations were 5 mg ficin and 10 mg bromelain per g of freeze-dried biceps femoris muscle. The buffers were: pH 2–6, 0.25*M* citrate; pH 6–8, 0.25*M* phosphate; and pH 8–10, 0.25*M* borate.

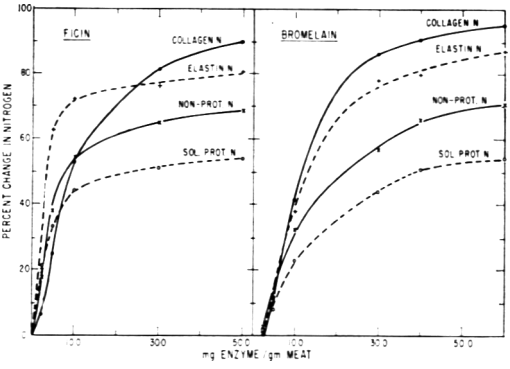

Fig. 2. Effect of enzyme concentration on extent of action of ficin and bromelain on beef proteins held at pH 5.6 and 60°C for 60 min.

ficin, papain, and bromelain on collagen was near pH 3 at 35°C, and that this was due to denaturation of collagen by acid followed by subsequent enzymatic attack (Hinrichs and Whitaker, 1962). However, at 60°C, maximum activity of ficin on purified collagen was at pH 7.0–7.8.

Change in pH has more influence on the extent to which collagen and elastin are attacked than on the extent to which the NaOH-soluble proteins are attacked by these enzymes. For example, there is respectively 10.6, 25.8, and 5.15% change in nitrogen of the soluble proteins, elastin, and collagen fractions at pH 4.0, and 26.2, 98.0, and 100% change in nitrogen at pH 7.0 in 60 min at 60°C when ficin is used. The pH of the rehydrated freeze-dried biceps femoris muscle of beef used in this work was 5.6, and subsequent experiments were carried out at this pH. It is not practical to add buffer to influence the pH of raw, fresh meat, but this may be done readily during the rehydration of freeze-dried meat.

Effect of enzyme concentration. Fig. 2 shows the effect of ficin and bromelain concentration on the disappearance of beef proteins in 60 min at pH 5.6 and 60°C. Over 80% of the collagen and elastin disappear at the high enzyme concentrations while only about 55% of the NaOH-soluble proteins fraction disappears. There appears to be some inhibition of bromelain by the meat substrate, as indicated by the intercept on the *X*-axis being at 2.5 mg rather than at the origin.

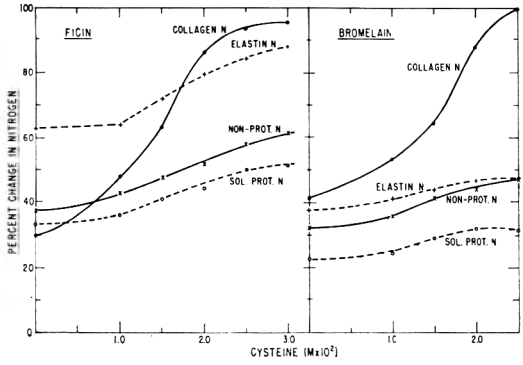

Fig. 3. Effect of cysteine concentration on extent of action of ficin and bromelain on beef proteins at pH 5.6 and 60°C for 60 min. Enzyme concentrations were 5 mg ficin and 10 mg bromelain per g of freeze-dried biceps femoris muscle.

Effect of activator concentration. Both ficin (Whitaker, 1957b) and bromelain (Murachi and Neurath, 1960) have been shown to be activated by sulfhydryl compounds. Gottschall and Kies (1942) showed that beef contained enough sulfhydryl groups to activate completely the papain used in their studies. Fig. 3 shows the effect of cysteine concentration on the hydrolysis of beef proteins by ficin and bromelain.

Addition of cysteine may have two effects on the extent of solubilization of the proteins. First, it may increase the percentage of active enzyme, and, second, it may in some manner affect the proteins so as to make them more easily solubilized by the enzyme. From a comparison of the data in Figs. 2 and 3 and the effect of cysteine on the hydrolysis of casein by ficin and bromelain (see Methods), one may separate these effects. The following results are obtained (in percentage change in nitrogen): for ficin, soluble proteins 50.0 and 51.4; elastin, 76.0 and 88.0; and collagen, 76.0 and 95.5; and for bromelain, soluble proteins 28.7 and 31.7; elastin, 54.6 and 46.5; and collagen, 62.0 and 100. The first value given for each protein fraction is the result one would expect if the only influence of the cysteine were in increasing the percentage of active enzyme, whereas the second value is the result obtained in the presence of the maximum amount of cysteine used. There is no effect of cysteine on the soluble proteins fraction. From the data with ficin it would appear there is some effect of cysteine on elastin, but this is not the case for bromelain. There is a large influence of cysteine on collagen as measured by both enzymes. Grant and Alburn (1960) reported that calcium salts, salicylates, arginine, creatinine, and guanidine all permitted trypsin, chymotrypsin, elastase, and endopeptidase from procarboxypeptidase A to solubilize collagen. There is also an increased solubilization of collagen by ficin in the presence of arginine (Hinrichs and Whitaker, 1962).

Fig. 3 shows the relative effectiveness of ficin and bromelain in hydrolyzing the proteins of beef muscle in the absence of added cysteine. In this study, 5 mg ficin and 10 mg bromelain per g freeze-dried beef were permitted to act for 60 min at 60°C and pH 5.6. On an equal-weight basis of enzyme (1 mg), bromelain is about as effective as ficin on collagen (4.13 vs. 5.92%), and ficin is about 3 times as effective as bromelain on the soluble proteins fraction (6.64 vs. 2.26%) and on elastin (12.5 vs. 3.78%). At higher enzyme concentrations, where the substrate is limiting, this difference is not noticeable. With the enzyme preparations used here and in the absence of cysteine and versene, casein is hydrolyzed 2.4 times more by bromelain than by ficin in 20 minutes. Wang *et al.* (1958) reported ficin to be twice as effective on elastin as bromelain.

In the absence of added cysteine, bromelain solubilizes collagen and elastin to about the same extent, and about twice as much

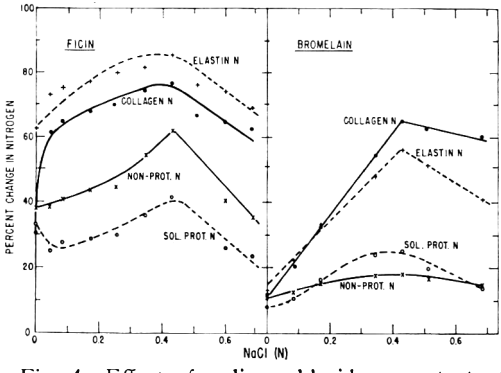

Fig. 4. Effect of sodium chloride on extent of action of ficin and bromelain on beef proteins at pH 5.6 and 60°C for 60 min. Enzyme concentrations were 5 mg enzyme per g of freeze-dried biceps femoris muscle.

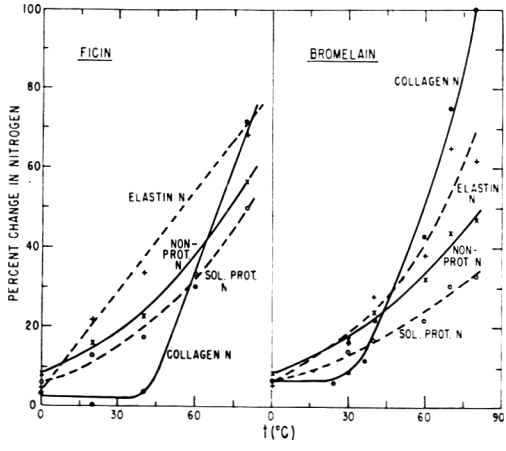

Fig. 5. Effect of temperature on extent of action of ficin and bromelain on beef proteins at pH 5.6 and 60 min. Enzyme concentrations were 5 mg ficin and 10 mg bromelain per g of freeze-dried biceps femoris muscle.

as the soluble proteins fraction. On the other hand, ficin solubilizes elastin to twice the extent of collagen, and the soluble proteins fraction is solubilized to a little larger extent than is collagen. This clearly demonstrates the difference in method of attack on beef proteins by these two proteolytic enzymes.

Effect of sodium chloride. As shown in Fig. 4, the maximum solubilization of beef proteins in 60 min at 60°C by both ficin and bromelain is near 0.43*N* sodium chloride (0.43 ionic strength); a further increase in sodium chloride concentration decreases the extent of solubilization. There is a large increase in the extent of solubilization of collagen by both ficin (120%) and bromelain (450%) as the sodium chloride concentration is increased up to 0.43*N*. Added sodium chloride also produced a significant increase in the extent of solubilization of purified collagen by ficin (Hinrichs and Whitaker, 1962). The effect of sodium chloride on denaturation of collagen has been discussed previously (Hinrichs and Whitaker, 1962). Sodium chloride also influences the extent of solubilization of elastin by bromelain (330%) more than it does with ficin (96%). It should be noted that ficin solubilizes elastin readily in the absence of salt. Influence of sodium chloride on the extent of solubilization of the soluble proteins fraction by either ficin or bromelain is less marked than on collagen and elastin.

Effect of temperature. Extent of action of ficin and bromelain on soluble proteins, elastin, and collagen fractions increases with temperature up to 80°C (Fig. 5). Even at 80°C there is no indication that heat denaturation of the enzymes is faster than the increase in solubilization of the beef proteins. Under other conditions the temperature optimum for ficin has been reported to be 63°C at pH 7.5 (Whitaker, 1957a); at pH 2.86 it is 35°C (Hinrichs and Whitaker, 1962). This indicates the degree of protection of the enzymes by the presence of the beef proteins.

It should be noted that there is a small but significant solubilization of the beef proteins by ficin and bromelain at 0°C. Tappel *et al.* (1956) found some hydrolysis by papain at this temperature. Change in temperature has more influence on the solubilization of collagen and elastin than it does on the solubilization of the soluble proteins fraction. There is very little solubilization of collagen by either ficin or bromelain below 40°C, but the extent of solubilization of collagen increases very rapidly as the temperature is increased. Again, this points out the need for denaturation of collagen before it is attacked by ficin or bromelain (Hinrichs and Whitaker, 1962). There is a marked difference in the amount of elastin (21.6%) and collagen (0%) solubilized by ficin at 20°C. Apparently, in contrast to collagen, elastin does not have to be denatured before ficin or bromelain can solubilize it. Elastin is usually considered to be more resistant than collagen to the action of proteolytic enzymes as well as to acids and bases. However, elastin is solubilized much better than collagen by ficin and bromelain at temperatures that do not denature collagen.

ACKNOWLEDGMENTS

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The Influence of Post-mortem Glycolysis on Poultry Tenderness[a]

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SUMMARY

The minimization of post-mortem glycolysis by 1) subcutaneous injections of adrenaline, which eliminates muscle glycogen ante-mortem, 2) intravenous injections of sodium iodoacetate, which inhibits phosphoglyceraldehyde dehydrogenase, or 3) rapid cooking, has resulted in poultry meat that is tender without aging. Since these treatments accelerate rigor mortis, the elimination of post-mortem glycolysis eliminates the toughening associated with an acceleration of rigor mortis in normal birds.

A number of physical agents that accelerate the onset of rigor mortis in poultry have been shown to lead to increased toughness in the aged meat (de Fremery and Pool, 1959, 1960). These post-mortem treatments, which increase the rate of onset of rigor without affecting the ultimate pH, include freezing and thawing, heating, elevated temperature, excising or cutting the muscle, electron irradiation, and exhaustive electrical stimulation. As with other meats (Erdös, 1943, Bate-Smith, 1948), the onset of rigor mortis in poultry (as measured by a decrease in muscle extensibility) is accompanied by a decrease in adenosine triphosphate (ATP), glycogen, and pH. It is obviously important to know which of these three changes is directly related to the toughening. One experimental approach applicable to this question was developed by Radouco-Thomas *et al.* (1959), who prevented post-mortem glycolysis in several mammalian species with an ante-mortem subcutaneous injection of adrenaline. Under suitable conditions, this technique depletes the muscle glycogen prior to slaughter (Cori and Cori, 1928).

The present paper reports the effects on tenderness in young birds of preventing or minimizing post-mortem glycolysis by three independent techniques: 1) injections of adrenaline to eliminate muscle glycogen ante-mortem; 2) injections of sodium iodoacetate to inhibit glycolysis; and 3) very rapid cooking before glycolysis has had time to proceed very far. Results show that meat is tender when it is cooked in the absence (or before the occurrence) of post-mortem glycolysis.

EXPERIMENTAL METHODS

Chemical determinations and evaluations of toughness were made on the pectoralis major. Tissue extracts for the determination of ATP were prepared by homogenizing 2 g of muscle in a blender with 20 ml of 5% trichloroacetic acid (TCA) and filtering. ATP was determined by the method of Griswold *et al.* (1951), except that the labile phosphate from ATP was separated by precipitation as $MgNH_4PO_4$ after hydrolysis in 1*N* H_2SO_4 for 15 min at 100°C. ATP-phosphorus values are reported as a percentage of total TCA-soluble phosphorus (TSP). Muscle pH was determined on a 15-g sample of muscle, homogenized in a blender in 30 ml of 0.005*M* sodium bromoacetate.

In all experiments in which whole chickens or turkeys were used, birds were processed under simulated commercial conditions (stunned by electric shock, bled 2 min from an outside neck cut, and scalded 60 sec at 53°C or 30 sec at 60°C, picked mechanically with minimum force, and

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Table 1. Effect of adrenaline injection on the tenderness of chicken aged for various times.

Experiment no.	Aging time: During processing (hr)	Aging time: In ice chill (hr)	Av. shear force: Adrenaline-treated (lb)	Av. shear force: Control (lb)	Level of significance of difference (%)
1	0.6	0	10	17	0.1
	0.6	24	6	6	.....
2	0.5	0	10	27	0.1
	0.7	1	7	25	0.1
	0.6	2	8	11	2
	0.6	24	.....	5	.....

eviscerated immediately). The relative toughness of cooked meat was determined with a Warner-Bratzler shear-force apparatus.

RESULTS

Effect of adrenaline on post-mortem biochemistry and tenderness. Initial studies showed that the following treatment with adrenaline depresses muscle glycogen in fryer chickens to about 5% of its normal level and therefore markedly modifies the post-mortem biochemistry. Briefly, the treatment consists of injecting a 24-hr-fasted bird subcutaneously with 1 mg per kg of adrenaline and then fasting the bird for an additional 16 hr before slaughter. Fig. 1 presents the results from a typical experiment. It is evident that ATP disappeared more rapidly in the adrenaline-treated bird than in a control fasted in the same way. The time required for the ATP concentration to fall to 50% of its initial value was reduced from 3.9 to 0.6 hr. As expected from the relation between ATP level and rigor, the onset of rigor occurred sooner in the adrenaline-treated birds.

This treatment also has a marked effect on the tenderness pattern, as shown by two tests on fryer chickens and one on light turkeys.

The results of the experiments with chickens are presented in Table 1. Each figure represents the average of 6–8 birds. The birds were frozen after processing or after the indicated aging time, and then sawed in half. One half from each bird was thawed rapidly in tap water, and used in determining the pH of the pectoralis major muscle. The other half was cooked from the frozen state in vegetable oil at 130°C for determination of the shear force of the pectoralis major. The pH serves as a check on the effectiveness of the treatment. In experiment 1, the average pH (± standard deviation) of all control birds was 5.80 (±0.09) whereas that for the adrenaline-treated birds was 6.56 (±0.10). In experiment 2, the average pH (± standard deviation) of all control birds was 5.71 (±0.14) whereas that for the adrenaline-treated birds was 6.51 (±0.17). These

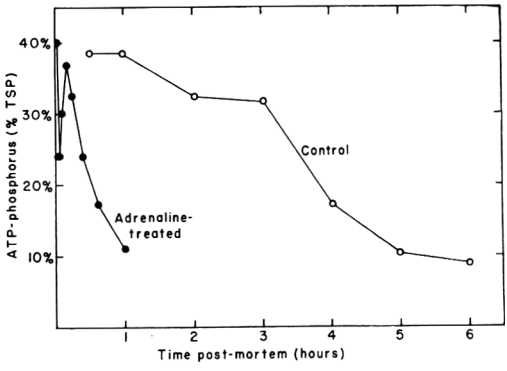

Fig. 1. Effect of adrenaline on the post-mortem breakdown of ATP at 16°C.

experiments show that the treated birds are almost as tender as the fully-aged control birds, even after minimal aging periods.

The experiment on turkeys was conducted with light turkey hens (6.0 lb average weight) in groups of eight birds each. Each bird in three groups received a subcutaneous injection of adrenaline (1.5 mg/kg). All groups were fasted for 18 hr and then processed as previously described. The pH and shear-force values of the muscles were determined as previously described, except that the cooking temperature was 110°C. The average pH (± standard deviation) of all control birds was 5.90 (±0.12) whereas that for the adrenaline-treated birds was 6.94 (±0.13).

Table 2 shows that the results are similar with

Table 2. Effect of adrenaline injection on the tenderness of turkey hens.

Aging time: During processing (hr)	Aging time: In ice chill (hr)	Av. shear force: Adrenaline-treated (lb)	Av. shear force: Control (lb)	Level of significance of difference (%)
0.6	0	8	22	0.5
0.6	1	7	30	0.1
0.6	2	8	27	0.5
0.6	24	.....	9	.....

Table 3. Effect of iodoacetate injection on the tenderness of cooked chicken meat.

Aging time		Av. shear force		Level of significance of difference (%)
During processing (hr)	In ice chill (hr)	Iodoacetate-treated (lb)	Control (lb)	
0.5	0	10	31	0.5
0.5	24	10	5	0.1

turkeys and chickens. Treated birds, aged for times inadequate for normal birds, were as tender as fully-aged control birds.

Effect of sodium iodoacetate on tenderness of chicken. Enzyme-inhibiting levels of sodium iodoacetate were used to determine how this reagent might affect tenderness, or, more specifically, the need for aging to achieve tenderness. This was done so that glycolysis could be eliminated a second way, namely, by inhibiting the enzyme phosphoglyceraldehyde dehydrogenase (Padieu and Mommaerts, 1960). Although this method of eliminating glycolysis differs in principle from the adrenaline method, both methods are accompanied by a more rapid onset of rigor and, of course, an ultimate pH higher than normal.

Table 3 shows that inhibition of post-mortem glycolysis by an intravenous injection of sodium iodoacetate is essentially as efficient in yielding tender meat without aging as is the prevention of glycolysis by adrenaline injection. The results were obtained on fasted chicken fryers injected with 200 mg iodoacetic acid per kg (as the sodium salt) 3–6 min before slaughter. The birds were frozen at times indicated in Table 3, and the pH and shear force values of the muscles were determined as previously described. Each figure in Table 3 represents the average of eight birds. The average pH (±standard deviation) of all control birds was 5.74 (±0.12) whereas that for the iodoacetate-treated birds was 6.76 (±0.25).

Tenderness of chicken muscle cooked very rapidly post-mortem. As a third method of avoiding or minimizing glycolysis, an attempt was made to cook normal chicken muscle so quickly that glycolysis would not have time to proceed very far. Processing steps, which in our other tests took about 30–40 min, were changed so that the interval between throat cut and cooking was 1½–2 min. After killing, the pectoralis major muscles were excised rapidly. One muscle from each bird was clamped between metal plates and placed immediately in boiling water, where it reached an internal temperature of 85°C in 3.1 min. The muscles were cooked for 30 min, and shear values were then determined on a one-inch strip from each muscle. These shear values were compared with shear values for paired control muscles held for 1 hr before cooking. The results (Table 4) clearly show that chicken muscle is more tender if cooked quickly, immediately post-mortem, than if aged for 1 hr. Quantitatively, this result is not so clear-cut as those from the first two methods used to avoid glycolysis. That is, fully-aged muscle would be still more tender than the 2-min muscle, possibly having a shear value in the range of 4–7 lb. However, some glycolysis undoubtedly occurred even in the short processing and cooking times employed.

The report of Koonz *et al.* (1954) on the tenderness pattern of chicken muscle under a variety of treatments contains data indicating that unaged quickly-cooked muscle is more tender than muscle aged 1 or 2 hr.

DISCUSSION

The results described in this paper on the metabolic changes accompanying rigor mortis (ATP breakdown, glycogen breakdown, and pH decrease) show that a rapid onset of rigor in young birds does not necessarily result in toughness. In the three conditions used to eliminate or minimize post-mortem glycolysis (adrenaline injection, iodoacetate injection, very rapid cooking), rapid disappearance of ATP and consequent rapid onset of rigor were not accompanied by toughness. Hence, rapid rigor and rapid disappearance of ATP have been eliminated as causes of toughening. It is not known whether a rapid rate of glycogen breakdown, a rapid rate of pH decrease (lactic acid formation), or some other post-mortem change related to glycolysis induces toughness when normal muscle goes into rapid rigor. However, we may speculate that an accelerated rate of formation of lactic acid is involved since acids do affect the stability of proteins. In this regard, Wismer-Pedersen (1959) showed a decreased protein solubility in pork when post-mortem glycolysis is accelerated, even though the

Table 4. Effect of *very* short aging period on tenderness of chicken.

Mean shear force (12 birds)	
Aged 2 min	= 12.4 lb
Aged 60 min	= 17.2 lb
Mean pair difference	= 4.8 lb*
Standard error	= 1.41 lb

* Difference in tenderness significant at the 1% level.

ultimate pH is normal.

In our experiments, glycolysis was minimized or eliminated primarily to study the effect of such treatments on the mechanical properties of cooked meat. Since the indirect action of adrenaline or sodium iodoacetate was to increase the ultimate pH, flavor may have been harmed. This possibility was studied for adrenaline by presenting cooked samples of treated and untreated chicken to a panel of trained judges. The very few adverse flavor comments received were divided about equally between control and treated birds. Thus, elevation of the ultimate pH by adrenaline appears to have little or no effect on meat flavor.

The pattern of tenderization in poultry is such that meat cooked a few minutes after death is more tender than meat allowed to age for one hour before cooking. With the normal occurrence of glycolysis, the meat rapidly becomes tough and remains tough until the aging process, in some unknown manner, renders the meat tender again. The transient period of initial tenderness is difficult to demonstrate in poultry since the very act of cooking accelerates the metabolic processes in meat. A period of initial tenderness appears to be more easily demonstrated for beef. Paul *et al.* (1952) showed that rapidly cooked meat was toughest when held for 24 hr before cooking, whereas meat held for 1 hr was as tender as meat held for 144 hr. Slowly cooked meat, on the other hand, was toughest immediately after slaughter. Poultry is apparently similar to beef in post-mortem tenderization, although the times are much shorter.

The prevention of post-mortem glycolysis by injecting adrenaline or sodium iodoacetate or by cooking meat so soon that glycolysis has not had time to proceed very far, provides evidence that glycolysis causes toughness. It also appears definite that the faster the glycolysis the greater the toughness. Evidently this influences the normal post-mortem changes in the muscle fibers. Perhaps it increases the degree of inter- and intramolecular bonds that change the muscle from an elastic to an inelastic fiber. The specific involvement of glycolysis and the possible role of lactic acid in toughness development are being studied further.

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The Major Anthocyanin Pigments of Vitis Vinifera Varieties Flame Tokay, Emperor, and Red Malaga

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SUMMARY

The table-grape variety Flame Tokay contains cyanidin-3-monoglucoside as the principal skin pigment, 82% of the total pigment, with no evidence for the presence of malvidin-3-monoglucoside. Skin pigment compositions for the Red Malaga and Emperor varieties were also determined.

INTRODUCTION

Vitis vinifera table-grape varieties Flame Tokay, Emperor, and Red Malaga are extensively grown in the great Central Valley of California. The Flame Tokay is essentially confined to an area in and around Lodi, where soil, climate, and perhaps other factors cause it to develop a particularly attractive light-red color. The Emperor and Red Malaga are grown in the more southern parts of the Central Valley and, though lightly pigmented, are usually somewhat darker than the Flame Tokay.

Rankine *et al.* (1958), from their survey of the pigmentation of red grape varieties, noted that the principal pigment band of Flame Tokay, Emperor, and Red Malaga had the same R_f as band 5 of the normal red vinifera pattern, but that it so differed in color as to suggest that it was a different pigment. This paper presents identification of the major anthocyanin pigments of these three table-grape varieties.

EXPERIMENTAL MATERIALS

Grapes. Grapes were harvested at maximum color development from the Davis experimental vineyards of the Department of Viticulture and Enology, University of California, Davis. The Flame Tokay was taken from the crops of 1959 and 1960, and the Emperor and Red Malaga from the 1961 crop.

Chromatographic equipment. Whatman Nos. 1 and 3 MM papers in sheets 46 by 57 cm were used in a Reco Chromatocab, Model A, for pigment separations. Glass jars and smaller sheets of paper were used for analytical procedures. Color densities of bands on paper strip chromatograms were measured with a Beckman Spinco Analytrol, Model RB, using interference filters to pass light at 500 mμ.

Solvent systems for paper chromatography were the following:

BAW	*n*-butanol : acetic acid : water, 4 : 1 : 5 by vol. (upper phase) (Bate-Smith, 1950).
BuHCl	*n*-butanol : 2*N* HCl, 1 : 1 by vol. (upper phase) (Harborne, 1958b).
2*N* HCl	(Bate-Smith, 1950).
Bu : pyridine	*n*-butanol : pyridine : water, 6 : 3 : 1 by vol. (Endo, 1959).
Benz : acetic	benzene : acetic acid : water, 2 : 2 : 1 by vol. (upper phase) (Bray and Thorpe, 1954).
EtOAc : acetic	ethyl acetate : acetic acid : water, 9 : 2 : 2 by vol. (Pridham, 1957).
Forestal	water : acetic acid : HCl, 10 : 30 : 3 by vol. (Harborne, 1958b).
Bu : ethanol	*n*-butanol : ethanol : water, 5 : 1 : 4 by vol. (upper phase) (Heftmann, 1961).

Chromogenic spraying reagents for location of sugars and phenolic compounds on paper chromatograms were:

Partridge's reagent (1949)—0.93 g aniline and 1.66 g phthalic acid dissolved in 100 ml of 95% ethanol. Papers sprayed with this reagent were dried in a fume hood and then oven heated 3 hr at 80°C.

Pauly's reagent—5 ml of a 1% solution of sulfanilamide in 10% HCl and 5 ml of 5% $NaNO_2$

[a] Taken from an M.S. thesis by Minoru Akiyoshi, University of California, Davis, 1962.

were mixed, allowed to stand 1 min, and made to 50 ml with *n*-butanol. After one hr the butanol phase was separated and used as the spray reagent. Sprayed papers were nearly dried in the fume hood and then sprayed with one-half saturated Na_2CO_3 solution.

EXPERIMENTAL PROCEDURES

Isolation of pigment from grapes. The grapes were washed in distilled water, dried, and peeled with a scalpel. Skins were washed repeatedly in distilled water while being rubbed between the fingers in order to remove adhering pulp. The clean skins were dried between layers of cheesecloth and stored in polyethylene bags under a nitrogen atmosphere at −4°F.

Pigment was extracted by pulping the skins from 6 kg of grapes for 2 min in a Waring blender with 200 ml of *n*-butanol, 50 g NaCl, and 1 ml conc. HCl. The purée was stored for two days at −4°F, and the phases were then separated. The aqueous pulp phase was extracted with three more portions of *n*-butanol, and the extracts were combined and stored at −4°F to induce separation of an aqueous phase. To the butanol phase were added two volumes of petroleum ether, which caused the separation of a concentrated aqueous pigment-containing layer.

Paper chromatographic purification of crude pigment. The crude pigment concentrate was purified with the bar chromatogram technique. Five successive 0.25-ml portions were streaked along the long dimension of a large sheet of Whatman 3 MM paper, and 8 such papers were developed in the Chromatocab at one time using the BAW solvent, descending, for 22 hr at room temperature. After drying in a fume hood, the papers were cut longitudinally so that separated pigment bands could be eluted into separate receivers. The eluting solvent was absolute methanol containing 3 drops conc. HCl per 100 ml. The eluted pigment solutions were concentrated under reduced pressure and nitrogen atmospheres to very small volumes, and were then taken up into *n*-butanol. On the addition of two volumes of petroleum ether and cooling, a small, pigment-containing second phase separated. This pigment concentrate was chromatographed a second time, using a procedure identical with that followed in the first chromatographing except that the papers were dried until no acetic acid odor was evident. Final elution of the pigment was then conducted.

Quantitative estimation of pigments. For quantitative estimations of the relative amounts of individual pigment compounds in each of the three varieties, aliquots of the crude pigment concentrates were bar chromatographed on 10×46-cm sheets of Whatman No. 1 paper, cut vertically into strips 4.5 cm wide, and color densities were measured in the Analytrol. To assure comparable results, each strip was steamed briefly and fumed with conc. HCl vapors before examination in the Analytrol. It was necessary to apply a correction factor to the value for the band-4 pigment because of the response of the Analytrol to this band (Albach, 1959, 1960).

Partial hydrolysis of the Flame Tokay main pigment. The techniques of Abe and Hayashi (1956) and of Harborne and Sherratt (1957) were followed in principle for hydrolysis of the anthocyanin. One ml of the methanolic solution of rechromatographed pigment was placed in a 12-ml centrifuge tube and taken to dryness under a current of nitrogen. Three ml of 10% HCl solution were added, a small cold-finger condenser was mounted in the upper portion of the tube, and the assembly was placed in a boiling-water bath. At 10-min intervals during 1 hr, aliquots were taken for spotting on each of three different chromatographic papers. The individual papers were developed with BAW, BuHCl, or 2*N* HCl.

Identification of sugar moiety. Five mg of nitrogen-dried pigment were hydrolyzed by heating under reflux for 1 hr in 1 ml of 10% HCl. After cooling, the excess HCl was just neutralized by adding small portions of Duolite A-3 (OH^-) resin. The sugar was recovered by repeated extraction of the mixture with absolute methanol. The separated methanolic solution of sugar was concentrated and spotted on Whatman No. 1 chromatographic paper along with the known sugars: xylose, rhamnose, glucose, fructose, and galactose. Separate papers were developed in BAW and Bu:pyridine. The sugar spots were made visible with Partridge's reagent.

Alkaline degradation. The technique of Karrer and Widmer (1927) was followed in its essentials for the pigment degradation. To 45 mg of nitrogen-dried pigment in a test tube was added 2 ml of hot 15% $Ba(OH)_2$ solution. With a current of nitrogen passing through the upper part of the tube, the contents were refluxed for 1 hr. At this time, small additions of solid $Ba(OH)_2$ were made until no undegraded pigment remained. (Undegraded pigment was detected by the appearance of a red color on acidification of a drop of the reaction mixture.) After cooling, the reaction mixture was acidified with conc. HCl to the point of $BaCl_2$ precipitation. The mixture was then extracted with seven successive 1-ml portions of ether, which were combined. The extracted degradation products were investigated chromatographically on Whatman No. 1 paper with the solvent systems: BAW, Benz:acetic, and EtOAc:acetic. Spotted on the same papers as knowns

were: gallic acid, syringic acid, protocatechuic acid, and phloroglucinol.

Spectra of the Flame Tokay pigment. The Beckman DK2 spectrophotometer was used to record the spectra of the Flame Tokay anthocyanin, anthocyanin, and pigment-aluminum complexes in the range of 400–700 mμ. Pigments were carefully purified and dissolved either in absolute methanol with 0.01% HCl or in absolute ethanol with 0.01% HCl. The spectral shift on the addition of $AlCl_3$ was measured by adding 3 drops of a 5% solution of the salt in absolute ethanol to the cuvette, mixing, and recording the spectrum at 5-min intervals up to 15 min.

RESULTS AND DISCUSSION

Chromatography of the pigment from skins of Flame Tokay, Emperor, and Red Malaga yields chromatograms in which the majority of the pigment is concentrated in bands 4 and 5 of the numbering system of Rankine *et al.* (1958). Band 4 was shown by Albach *et al.* (1962) to be peonidin-3-monoglucoside. Band 5, in the majority of red wine vinifera grapes, has been identified as malvidin-3-monoglucoside, but. in the case of the table grape Flame Tokay in particular, the color of the pigment with R_f corresponding to band 5 is different enough to suggest that it is not malvidin-3-monoglucoside.

Partial hydrolysis and chromatography of the band-5 pigment of Flame Tokay gave results as summarized in Table 1. The fact that only one more pigment was obtained by partial hydrolysis indicates that the original pigment was a monoglycoside.

Table 1. R_f values of Flame Tokay band-5 pigment.

	BAW	BuHCl	2N HCl
Original pigment	0.19	0.22	0.12
Hydrolyzed pigment	0.46	0.70	0.03

Chromatography of the sugar obtained from complete hydrolysis of the Flame Tokay band-5 pigment gave results as listed in Table 2. It is evident from the data that the sugar attached to the anthocyanidin is glucose.

The nature of the anthocyanidin was established by alkaline microdegradation of a sample of the Flame Tokay band-5 pigment. The results of chromatography of the products of the degradation are summarized in Table 3. The data indicate that the products of alkaline degradation of the pigment are phloroglucinol and protocatechuic acid, the products expected from the degradation of cyanidin. It is thus established that the Flame Tokay band-5 pigment is a cyanadin monoglucoside.

Table 2. R_f values of sugar from hydrolyzed Flame Tokay band 5.

	Ascending		Descending	
	Bu-pyridine	BAW	BAW[a]	Bu-ethanol
Unknown	0.17	0.06	0.74	0.10
Glucose	.18	.06	.76	.10
Galactose	.15	.08	.67	.09
Fructose	.24	.15	1.00	.14
Xylose	.31	.18	....	....
Rhamnose	.47	.31	....	....

[a] Solvent front ran off bottom of paper. Values are relative to fructose.

The point of attachment of the glucose to the anthocyanidin molecule is assumed to be on the number-three carbon atom because the spectral characteristics of the Flame Tokay pigment band 5 are in agreement with those given by Harborne (1958a) for cyanidin-3-monoglucoside (Table 4). It is observed, in addition, that the Flame Tokay pigment and its aglycone do not fluoresce under ultraviolet light. A sample of cyanidin-3,5-diglucoside extracted from the petals of the Chrysler Imperial rose, and one of the three products obtained from it by partial hydrolysis (presumably cyanidin-5-monoglucoside), do fluoresce brightly. Since Harborne (1958b) and Abe (1956) have correlated bright fluorescence in anthocya-

Table 3. R_f values of degradation products of Flame Tokay band 5.

Compound	Benz-acetic	BAW	EtOAc-acetic	DSA color
Unknown A	0.04	0.70	0.82	yellow
Unknown B	.40	.80	.92	brown-tan
Phloroglucinol	.05	.70	.84	yellow
Protocatechuic acid	.40	.80	.94	brown-tan
Gallic acid	.07	.63	.76	tan
Syringic acid	.81	.87	.95	red
Vanillic acid	.83	....	.97	orange
p-OH-Benzoic acid	.45	....	.98	yellow

Table 4. Spectral characteristics of tokay pigment compared with Harborne's data[a] for cyanidin-3-monoglucoside and cyanidin.

Methanolic 0.01% HCl	Tokay pigment	Cyanidin-3-monoglucoside[a]	Tokay anthocyanidin	Cyanidin[a]
$E_{440}/E_{max.}$	0.22	0.22	0.19	0.19
$AlCl_3$ shift	+	+	+	+
λ max.	527	525	537	535
Ethanolic 0.01% HCl				
λ max.			545	545

[a] Harborne (1958a).

nins with the presence of sugar on the number-five carbon, lack of fluorescence in the Tokay cyanidin-monoglucoside presumably establishes it as cyanidin-3-monoglucoside. Table 5 gives data obtained in chromatography of the Tokay and Chrysler Imperial rose pigments.

The identities and relative quantities of the pigments in the three grape varieties Flame Tokay, Emperor, and Red Malaga were determined by measurements of the pigment densities on chromatograms made with the BAW solvent and with the Forestal solvent. The values for peonidin were corrected to be comparable with those of cyanidin and malvidin using the technique developed by Albach (1959, 1960) for the Analytrol. The compositions are listed in Table 6. It is of interest to note that Ribéreau-Gayon (1959) found the Muscat Hamburg to be different from all of the other vinifera varieties studied in that it had pigment of which 65% was cyanidin and peonidin glucosides. In comparison, in the present investigation, the Flame Tokay is found to contain 89%, the Red Malaga 84%, and the Emperor 67% of these same pigments. In a previous investigation in this laboratory (Albach, 1960) the Flame Muscat was found to have pigments 95% of which were cyanidin and peonidin glucosides.

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Table 6. Pigment composition of table grapes.

Variety	Pigment (%)				
	Low R_f, BAW unknown	Cyanidin-3-monoglucoside	Malvidin-3-monoglucoside	Peonidin-3-monoglucoside	High R_f, BAW unknown
Tokay	6	82	0	7	5
Emperor	10	10	16	57	5
Red Malaga	9	60	2	24	5

Table 5. R_f values for Tokay and Chrysler Imperial rose pigments and partial hydrolysis products.

	H_2O:HCl:: 97:3	BAW	BuHCl	Appearance	
				Visible	u.v.
Tokay cyanidin					
3-glucoside	0.06	0.19	0.22	mauve	dull
aglycone	.02	.44	.70	pink	dull
Chrysler rose cyanidin					
3,5-diglucoside	0.13	0.08	0.07	red	red fluorescence
3-glucoside	.06	.19	.21	mauve	dull
5-glucoside	.06	.25	.34	orange	pink fluorescence
aglycone	.01	.44	.70	pink	dull

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The Chemical Composition of Maple Sugar Sand[a]

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SUMMARY

The gritty material obtained by filtering commercial maple sirup was analyzed to determine its composition and to relate its composition to the amount of sugar sand deposited to determine the factors responsible for the formation of sugar sand. The samples, taken over a two-year period, contained calcium, malic acid, and undetermined material (probably silica) as the major constituents. The calcium, malic acid, and calcium malate content gave highly significant positive correlations with the amount of sugar sand formed, whereas the percentage of undetermined material gave a negative correlation. There was also evidence that the malic acid content was more critical in the formation of sugar sand than the calcium content. Highly significant negative correlations were obtained between the percent sugar sand deposited and the iron, copper, and boron content. Further, these constituents also gave highly significant negative correlations when compared with the calcium content of the sugar sand. The presence of potassium, magnesium, and molybdenum appeared to have little effect on the formation of sugar sand. The nonvolatile organic acids present in sugar sand were determined by paper chromatography. Results showed that sugar sand contains malic, citric, succinic, fumaric, and three unidentified organic acids.

INTRODUCTION

Sugar sand, niter, or malate of lime is an insoluble substance formed when the sap of the sugar maple tree (*Acer* spp.) is concentrated to maple sirup. Some of this sandy material is deposited on the evaporating pan, while most of the sugar sand stays suspended in the sirup and must be removed by sedimentation or filtration to meet federal as well as some state standards of clarity for table maple sirup.

Willits (1958) reported that the quantity and color of sugar sand are not always the same, for the constituents of the sap vary with the year, run, and sugar bush location. He also reported that the species of maple will affect the quantity of sugar sand deposited. Gallander (1961) found that sugar sand ranged in color from almost white to black, and further, these extreme color changes were noted in different runs from the same sugar bush during the same season.

Salts of organic acids are generally agreed

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to be important constituents of maple sugar sand. Calcium malate has been recognized as the principal organic salt in sugar sand (Noller, 1958; Shead, 1952; Willits, 1958). Nelson (1928) separated and identified the following organic acids in maple sugar sand: l-malic, formic, acetic, fumaric, succinic, and citric acids. More recently Willits (1958) reported that maple sirup contained the following nonvolatile organic acids: malic, citric, succinic, fumaric, glycolic or dihydroxybutyric, and seven unidentified acids.

Some of the first investigators of sugar sand found varying amounts of calcium and malic acid in this material. Huston and Bryan (1899) reported that the sugar sand samples they examined contained 12.89% calcium and 20.86% malic acid. Warren (1911), in a more detailed analysis, determined some 10 constituents of sugar sand. He reported that the constituents varied only within narrow limits, and his results showed the concentration of calcium to be 17.16% and malic acid to be 51.48% An investigation by Snell and Lockhead (1914) of six sugar sand samples that were washed and air dried showed that the insoluble material contained 65–85% calcium malate, 6–18.5% silica, minor quantities of magnesium, manganese, phosphorus, and iron, and 10–17% undetermined material.

In the present investigation, the amount of sugar sand formed in a given amount of maple sirup was related to its chemical composition. The chemical constituents were further examined and their relationship to one another and to the quantity of sugar sand deposited was studied. The results presented represent data from the analysis of sugar sand from sirups made in two successive years.

MATERIALS AND METHODS

The sugar sand samples were obtained from commercial maple sirup producers located in northeastern Ohio. The samples from the 1960 season were from 19 producers, and the 1961 samples were from 10 producers. Each sample of sugar sand represented the deposit formed in at least 5 gallons of maple sirup. The samples were removed from the filters by scraping the sand into polyethylene bags and transporting to the Horticulture Laboratories in Wooster, where they were kept under refrigeration (32–36°F) until analyzed. Sugar sand was arbitrarily defined for this study as the material deposited on the filter minus the moisture content and total sugar content. The percent sugar sand for each run was determined by the following formula:

$$\% \text{ SS} = 100\ (WSS)/[(WMS) + (WFM)]$$

where SS = sugar sand, W = weight, MS = maple sirup, FM = material retained on the filter.

Preliminary investigations by Davis and Gallander (1959) showed that an attempt to remove the sirup from the sugar sand by washing could dissolve and remove some of the constituents of the sugar sand. All samples, therefore, were analyzed without attempting to remove the sirup portion.

The moisture content was determined on the "as received" sugar sand samples with the A.O.A.C. vacuum oven method (A.O.A.C., 1950). The sugar content was determined on the "as received" samples from both seasons and on the dried samples of the 1961 season. The sugars were inverted to reducing sugars by acid hydrolysis and determined by the A.O.A.C. procedure (A.O.A.C., 1950) and the results calculated as percent sucrose. pH was determined with a glass-electrode Beckman Zeromatic pH meter. The free acid content was determined by titrating 1.0 g of sample in 50 ml of distilled water to a pH of 8.3 with 0.01N NaOH.

The total calcium, magnesium, and potassium content of the sugar sand samples was determined by flame photometry with a Beckman DU spectrophotometer with photomultiplier and flame attachment. A 1-g dried sample was ashed at 490°C for 7 hr and then cooled and moistened with distilled water. The ash was treated with 10-ml of conc. HCl and evaporated slowly to dryness. A second 10-ml portion of conc. HCl was added, and, after again evaporating to dryness, the residue was dissolved in 20 ml of 1.5N HNO_3, transferred to a 100-ml volumetric flask, and made to volume. After the insoluble material had settled out, the solution was analyzed. The flame spectrum was measured at the following wavelengths: calcium, 422 mμ; magnesium, 285.2 mμ; and potassium, 767 mμ.

The total manganese, boron, phosphorus, molybdenum, copper, and iron content was determined by spectrographic analysis with a B & L 1.5-meter replica grating spectrograph. The samples were prepared for the analysis by ashing at 490°C for 7 hr. Absorption readings were also recorded for the lines representing aluminum and silica, and results relating to these two constituents are based on the absorption percent rather than on the actual content of aluminum or silica in the sample.

Malic acid was determined by a modification of

the method described by Willits *et al.* (1958). Since a major portion of the malic acid was present in the form of calcium malate, it was necessary to remove the calcium from the malic acid component. One g of dried sugar sand was dissolved in 125 ml of distilled water and centrifuged to removed insoluble material. The supernatant liquid was passed through a Dowex resin column (17 × 0.75 in.) containing 50 ml of cation resin (H^+) at a rate of 2 to 3 ml per min. The resulting effluent was diluted to 250 ml by washing the column with approximately three bed volumes of water. The remainder of the procedure was the same as that of Willits *et al.* (1958).

The total organic acids content was determined by transferring 200 ml of the cation-free sugar sand solution that was used in the malic acid determination to a Dowex 1-8X (formate) resin column (17 × 0.75 in.) and allowing to pass through the column at a rate of 2 ml per min. The column was washed with distilled water until the eluate gave a negative anthrone test for carbohydrate. The acids were eluted off the resin column by passing approximately 200 ml of 10.N formic acid through the column at a rate of 1 to 2 ml per min. A 5-ml sample was transferred to a test tube and evaporated to dryness by passing filtered air over the surface of the liquid at 40°C. Five ml of CO_2-free water was then added to the test tube, and the solution was titrated with 0.01.N NaOH using phenolphthalein as an indicator. The percent total acids was calculated using the malic acid factor.

The presence of nonvolatile organic acids in the sugar sand samples was investigated by paper chromatographic methods. A 10-g dried sample was dissolved in 500 ml of distilled water and filtered to remove the insoluble material. Then the filtrate was passed through a Dowex 50 (hydrogen) cation exchanger. After the column was washed with 150 ml of distilled water to remove all free acids, the acids were absorbed on a Dowex 1-8X (formate) resin. This column was washed with distilled water to remove the sugars as shown by a negative anthrone test for carbohydrates. The acids were eluted from a column with 200 ml of 10.N formic acid, and the effluent was concentrated by boiling until the test papergrams would give the best resolution. The acid chromatograms were prepared by spotting on Whatman No. 1 paper and were developed by ascending chromatography with the following three solvent mixtures: I) *n*-butanol–acetic acid–water (4:1:5 v/v); II) *n*-butanol–formic acid–water (10:2:5 v/v); and III) *n*-pentanol–5.N formic acid (1:1 v/v). The acids were located by spraying with a 0.04% solution of bromophenol blue in 95% ethanol at pH 6.9.

Data from the foregoing analysis were treated by statistical procedures described by Snedecor (1956).

RESULTS AND DISCUSSION

Chemical constituents causing the formation of sugar sand. Tables 1 and 2 show complete chemical analysis of sugar sand obtained from maple sirups made in 1960 and 1961. Table 3 shows the correlation coefficients of the chemical constituents as compared to the amount of sugar sand formed. Although there is a highly significant positive correlation between the amount of sugar sand formed and the percent calcium, total malic acid, total organic acids, and calcium malate for the 1960 and 1961 sirups, the correlation coefficients are not as high as was expected. The highly significant correlation between the quantity of sugar sand formed and total organic acid content is actually of little importance since in all samples the malic acid is by far the major organic acid and there is no significant correlation between the amount of sugar sand deposited and the percentage of other organic acids.

Some of the heavy metals—iron, copper, and aluminum in particular—showed significant negative correlations with the quantity of sugar sand formed. The correlation coefficients for aluminum and silica are questionable, since the amounts were not measured quantitatively, so that correlations were based only on their spectrophotometer absorption values.

Thus, the only substances that had highly significant positive correlations with the amount of sugar sand formed were calcium, total malic acid, and calcium malate. These results support the theory that calcium malate is a major constituent affecting the formation of sugar sand.

Although the presence of calcium and malic acid in the sap will, to some extent, determine the amount of sugar sand deposited, the actual amount of these two constituents (referred to as calcium malate in the tables) in the sugar sand as it comes from the filter is less than 50% (Tables 1 and 2). The sugar content of these samples, as shown in Table 2, was at least 30% in all samples, and in some it amounted to more

than 80%. Most maple sirup producers agree that as the season progresses, the amount of sugar sand formed increases. If this is true, the percentage of calcium and malic acid would also be expected to increase accordingly. Table 4, however, shows that the probability of having more sugar sand deposited in the last run as compared to the first run was very small in the 1960 samples and highly probable for those samples taken from the 1961 sirups. Further, in the samples from the 1961 season, although the amount of sugar sand formed in sirups made from the last run of sap was significantly higher than that in sirup from the first run of sap and the malic acid content also showed the same trend, the difference in the calcium content between the two runs of sirup was not significant. These results indicate that the amount of malic acid contained in the sap could be more critical than the calcium content in determining the quantity of sugar sand formed during the evaporation of the sap to sirup. The data in Tables 1 and 2 show that the ratio of calcium content to malic acid content averages about 1:3, which

Table 1. Results of the chemical analysis of sugar sand (1960).

Sample code [a]	% sugar sand in run	% Ca	% Mg	% K	% total malic acid	% total organic acids	% acids other than malic	% calcium malate [b]
031	0.06	1.52	0.171	0.625	1.67	1.71	0.04	3.23
032	0.03	0.98	0.160	0.261	2.36	2.63	0.27	3.34
045	1.20	6.81	0.047	0.289	17.70	17.92	0.22	24.51
101	0.16	0.35	0.001	0.325	2.37	2.46	0.09	2.32
102	1.09	4.60	0.044	0.258	13.94	14.56	0.62	18.54
103	0.66	3.30	0.044	0.300	10.37	10.95	0.58	13.67
161	0.23	0.35	0.103	0.300	1.44	1.52	0.08	1.79
162	0.02	0.45	0.074	0.400	1.44	1.54	0.10	1.89
163	0.02	0.45	0.056	0.355	1.15	1.89	0.74	1.60
171	0.16	5.90	0.041	0.205	15.34	15.96	0.62	21.24
172	0.35	5.78	0.044	0.350	15.11	15.84	0.73	20.89
173	0.51	7.58	0.038	0.300	22.30	23.66	1.36	11.15
181	0.16	0.55	0.017	0.375	3.38	3.40	0.02	3.93
191	0.09	1.10	0.029	0.273	4.76	4.87	0.11	5.86
192	0.54	7.13	0.065	0.210	17.91	29.62	11.71	25.04
193	0.44	5.78	0.032	0.238	17.85	18.02	0.17	23.63
213	0.71	6.90	0.019	0.213	25.18	25.34	0.16	32.08
231	0.09	0.35	0.006	0.270	1.07	1.98	0.91	1.42
241	0.01	0.98	0.087	0.233	2.31	2.78	0.47	3.29
291	0.12	0.48	0.006	0.335	0.71	1.32	0.61	1.19
302	0.03	0.35	0.000	0.270	1.11	1.45	0.34	1.46
311	0.04	0.30	0.001	0.188	0.51	0.96	0.45	0.81
312	0.01	0.30	0.015	0.083	0.48	0.84	0.36	0.78
313	0.02	0.35	0.023	0.215	0.74	1.60	0.86	1.09
351	0.08	5.25	0.047	0.335	14.45	20.48	6.03	19.70
352	0.19	7.80	0.032	0.250	19.93	26.87	6.94	27.73
371	0.42	6.60	0.044	0.305	17.42	21.93	4.51	24.02
381	0.18	0.83	0.026	0.325	3.42	4.77	1.35	4.25
411	1.30	5.90	0.001	0.253	21.02	23.14	2.12	26.92
413	1.11	5.50	0.023	0.335	16.81	17.42	0.61	22.31
445	0.40	0.65	0.006	0.353	1.34	2.34	1.00	1.99
491	0.02	1.00	0.012	0.121	2.61	4.08	1.47	3.61
Range of values	0.01—1.30%	0.30—7.80%	0.000—0.171%	0.083—0.625%	0.48—25.18%	0.84—29.62%	0.02—11.71%	0.78—32.08%

[a] First two numbers designate the collaborator; the last number is the run number.
[b] Percent calcium plus percent total malic acid.

Table 2. Results of the chemical analysis of sugar sand (1961).

Sample code [a]	% sugar sand in run	pH	% Ca	% K	% Mg	% Mn	% P	ppm Fe	ppm Cu	ppm B	ppm Mo	% free acid	% total malic acid	% total organic acids	% acids other than malic	% undetermined material	% calcium malate [b]	% sugars of dried samples	% sugar sand of dried samples
041	0.66	6.80	3.75	0.148	0.018	0.16	0.08	347	48	5.5	0.30	0.15	8.80	9.24	0.44	12.91	12.40	73.77	26.23
046	1.42	6.85	6.41	0.148	0.025	0.18	0.20	169	21	8.1	0.79	0.13	17.20	19.82	2.62	10.91	23.48	62.31	37.69
051	0.28	7.20	0.61	0.205	0.025	0.17	0.32	677	123	8.8	0.30	0.07	0.76	0.81	0.05	17.20	1.30	80.66	19.34
057	0.64	6.80	5.45	0.158	0.018	0.16	0.10	178	11	3.8	0.30	0.16	14.25	14.51	0.26	13.25	19.54	66.35	33.65
121	0.06	7.00	0.54	0.210	0.014	0.14	0.16	520	82	6.4	0.25	0.15	1.20	1.63	0.43	11.57	1.59	85.74	14.26
125	0.05	6.80	0.72	0.213	0.011	0.19	0.31	830	65	8.3	0.79	0.20	1.43	1.74	0.31	14.68	1.95	82.14	17.86
171	0.42	6.85	6.15	0.241	0.022	0.11	0.18	1250	37	6.3	0.30	0.11	18.40	19.29	0.89	11.44	24.44	62.57	37.43
173	0.43	6.30	4.97	0.229	0.025	0.11	0.07	630	10	6.3	0.40	0.27	12.42	12.50	0.08	12.19	17.12	69.97	30.03
192	0.44	7.05	4.50	0.310	0.029	0.29	0.68	747	75	7.6	0.78	0.16	13.70	14.21	0.51	15.47	18.04	64.51	35.49
193	0.59	7.00	5.05	0.320	0.029	0.27	0.48	453	38	7.6	0.70	0.17	14.75	15.64	0.89	13.82	19.63	64.39	35.61
271	0.07	7.15	1.69	0.197	0.190	0.22	1.18	1025	143	23.0	0.88	0.13	2.85	2.97	0.12	34.16	4.41	59.39	40.61
272	0.77	6.75	10.91	0.146	0.039	0.16	0.57	254	52	8.3	0.30	0.37	38.87	40.68	1.81	13.59	49.41	33.91	66.09
281	0.36	6.70	6.35	0.380	0.018	0.07	0.12	67	15	6.8	0.17	0.12	17.56	19.05	1.49	8.28	23.79	65.80	34.20
283	0.54	6.75	5.20	0.320	0.025	0.06	0.03	38	7	5.5	0.17	0.10	15.93	16.52	0.59	6.94	21.03	70.99	29.01
361	0.12	6.80	3.95	0.245	0.018	0.12	0.48	213	41	5.0	2.46	0.27	11.06	12.01	0.95	13.10	14.74	70.08	29.92
362	0.64	6.85	4.87	0.280	0.014	0.07	0.19	138	16	5.1	0.17	0.23	13.94	15.33	1.39	8.32	18.58	70.99	29.01
411	0.41	7.15	5.25	0.224	0.036	0.07	0.24	148	15	4.6	0.40	0.12	15.94	16.22	0.28	8.39	21.07	69.73	30.37
413	0.98	7.00	4.96	0.237	0.029	0.09	0.22	178	15	3.4	0.30	0.15	14.50	14.58	0.08	8.86	19.31	71.11	28.89
431	0.18	6.90	1.69	0.270	0.036	0.07	0.16	860	40	7.2	0.17	0.20	7.56	9.30	1.74	13.00	9.05	75.54	24.46
434	0.26	6.60	3.03	0.250	0.025	0.06	0.17	788	35	5.5	0.30	0.29	10.31	11.44	1.13	11.47	13.03	73.61	26.36
Range of Values:																			
	0.05— 1.42%	6.30— 7.20 pH	0.61— 10.91%	0.146— 0.380%	0.011— 0.190%	0.06— 0.29%	0.03— 1.18%	38— 1250 ppm	7— 143 ppm	3.4— 23 ppm	0.17— 2.46 ppm	0.07— 0.37%	0.76— 38.87%	0.81— 40.68%	0.08— 2.62%	6.94— 34.16%	1.30— 49.41%	33.90— 85.74%	14.26— 66.09%

[a] First two numbers designate the collaborator; the last number is the run number.

[b] Percent calcium plus percent total malic acid minus percent free acid.

Table 3. Correlation coefficients between various chemical constituents and the amount of sugar sand formed (1960 and 1961).

Constituents correlated	Correlation coefficients 1960	1961
% sugar sand vs:		
% calcium	.629 **	.639 **
% potassium	−.049	−.263
% magnesium	−.192	−.219
% manganese		.044
% phosphorus		−.238
ppm iron		−.513 *
ppm copper		−.483 *
ppm boron		−.287
ppm molybdenum		−.195
aluminum content		−.535 **
silica content		−.414
% calcium malate	.689 **	.584 **
pH		−.087
% free acid		−.038
% total malic acid	.689 **	.562 **
% total organic acids	.611 **	.573 **
% acids, other than malic	.062	.434
% undetermined material		−.365

* Indicates significance at the 5% level.
** Shows significance at the 1% level.

further indicates that the amount of malic acid present could be a limiting factor in the formation of calcium malate.

The chemical composition of sugar sand. Table 2 shows the percentages of the various constituents of the dried samples from the 1962 season, which contained sugar sand and adhering sugar. Variations in the total sugar content of the samples caused differences in the sugar sand content, and since the adhering sugar could not be removed before analysis without the removal of some of the sugar sand constituents, this variation was mathematically eliminated by adjusting the values shown in Table 2 and expressing them on a sugar-free basis. This was done by dividing the percent of each constituent shown in Table 2 by the percent sugar sand of the dried sample and multiplying by 100. The adjusted values are given in Table 5.

These results are similar to those in Table 2 in that although there is a wide range of values for each constituent, the major components were calcium, malic acid, and undetermined material.

The correlation coefficients between the adjusted amount of chemical constituents and the percent sugar sand, shown in Table 6, were similar to those reported in Table 3 for the unadjusted values except that the negative correlations between iron, copper, boron, and the undetermined material become highly significant. The negative correlation coefficient between iron content and percent sugar sand (−.629) is higher than the positive correlation coefficient between malic acid content and percent sugar sand (.597) and is almost as high as the correlation coefficient between calcium content and percent sugar sand (.655).

Although there was no significant correlation between percent sugar sand and percent undetermined material before the values were adjusted (Table 3), after the values were adjusted to eliminate the effect of total sugars content there was a highly significant negative correlation (−.630). Since it has been previously reported (Snell and Lockhead, 1914) that the silica content in sugar sand is quite high, it could be assumed that most of this undetermined material is probably silica. The presence of silica in the sap apparently has little importance in the formation of sugar sand since the correlation is negative. This assumption was fur-

Table 4. Statistical comparison of various constituents in sugar sand between the first run of the season and the last run of the season.

	N	First run $\bar{x}$	Last run $\bar{x}$	D.F.	t	P
1960						
Amount of sugar sand deposited	8	0.27	0.37	7	1.14	.30
1961						
Amount of sugar sand deposited	10	0.30	0.63	9	3.63	.01
Calcium content	10	3.45	5.16	9	1.43	.20
Total malic acid content	10	9.78	15.41	9	3.66	.01

Table 5. Adjusted results of the chemical analysis of sugar sand (1961).

Sample code [a]	% sugar sand in run	% Ca	% K	% Mg	% Mn	% P	ppm Fe	ppm Cu	ppm B	ppm Mo	% free acid	% total malic acid	% total organic acids	% acids other than malic	% undetermined material	% calcium malate [b]
041	0.66	14.29	0.564	0.069	0.610	0.305	1323	183	21	1.1	0.57	33.55	35.23	1.68	48.93	47.27
046	1.42	17.01	0.393	0.066	0.478	0.531	448	56	21	2.1	0.34	45.64	52.59	6.95	28.93	62.31
051	0.28	3.15	1.060	0.129	0.879	1.654	3449	636	46	1.6	0.36	3.93	4.19	0.26	88.94	6.72
057	0.64	16.20	0.470	0.053	0.475	0.297	529	33	11	0.9	0.48	42.35	60.62	18.27	21.89	58.07
121	0.06	3.79	1.473	0.098	0.982	1.122	3647	575	45	1.8	1.05	8.42	11.43	3.01	81.11	11.16
125	0.05	4.03	1.193	0.062	1.064	1.736	4647	364	46	4.4	1.12	8.01	9.74	1.73	82.18	10.92
171	0.42	16.43	0.644	0.059	0.294	0.481	3340	99	17	0.8	0.29	49.16	51.54	2.38	30.55	65.30
173	0.43	16.55	0.763	0.083	0.366	0.233	2098	33	21	1.3	0.90	41.36	41.63	0.27	40.38	57.01
192	0.44	12.70	0.873	0.082	0.817	1.916	2105	211	21	2.2	0.45	36.80	40.04	1.44	43.57	49.05
193	0.59	14.18	0.899	0.081	0.758	1.348	1272	107	21	2.0	0.48	41.42	43.92	2.50	38.81	55.12
271	0.07	4.16	0.485	0.468	0.542	2.906	2524	352	57	2.2	0.32	7.02	7.31	0.29	84.13	10.86
272	0.77	16.51	0.220	0.059	0.242	0.862	384	80	13	0.5	0.56	58.81	61.55	4.74	20.56	74.76
281	0.36	18.57	1.111	0.053	0.205	0.351	196	44	20	0.5	0.35	51.35	55.70	4.35	24.01	69.57
283	0.54	17.92	1.103	0.086	0.207	0.103	131	24	19	0.6	0.34	54.91	56.95	2.04	23.63	72.49
361	0.12	13.20	0.819	0.060	0.401	1.604	712	137	17	8.2	0.90	36.97	40.14	3.17	43.78	49.27
362	0.64	16.79	0.965	0.048	0.241	0.655	476	55	18	0.6	0.79	48.05	52.84	4.79	28.46	64.05
411	0.41	17.29	0.738	0.119	0.230	0.790	487	49	15	1.3	0.40	52.49	53.41	0.92	27.42	69.38
413	0.98	17.17	0.820	0.100	0.312	0.762	616	52	12	1.0	0.52	50.19	50.47	0.28	30.37	66.84
431	0.18	6.91	1.104	0.147	0.286	0.654	3516	164	29	0.7	0.82	30.91	38.02	7.11	52.88	37.00
434	0.26	11.48	0.947	0.095	0.277	0.644	2986	133	21	1.1	1.10	39.07	43.35	4.28	43.21	49.45
Range of values:																
	0.05— 1.42%	3.15— 18.57%	0.220— 1.473%	0.048— 0.468%	0.205— 1.064%	0.103— 2.906%	131— 4647 ppm	24— 575 ppm	11— 57 ppm	0.5— 8.2 ppm	0.5— 8.2	0.29— 1.12%	3.93— 54.91%	4.19— 56.95%	0.26— 18.27%	20.56— 88.94%	6.72— 74.76%

[a] First two numbers designate the collaborator; the last number is the run number.

[b] Percent calcium plus percent total malic acid minus percent free acid.

ther supported by Gallander (1961), who analyzed the sap from a cooperator that has had no sugar sand formation in the past 15 years and found that silica was present in concentrations equivalent to that of other cooperators who had the usual sugar sand deposits.

None of the highly significant correlation coefficients between the amount of sugar sand deposited and the various chemical constituents are particularly high. However, there is a definite indication that in those sirups in which a large amount of sugar sand was deposited, the major constituents of the sugar sand were calcium and malic acid (presumably as calcium malate). In runs in which only a small amount of sugar sand was formed, the sugar sand was composed mainly of undetermined material (probably silica and extraneous material). It is quite possible that this deposition of undetermined material is fairly constant between runs and even between cooperators since its importance increased only as the amount of sugar sand formed in a given amount of sirup decreased.

Table 6. Correlation coefficients between the adjusted values of the chemical constituents and the amount of sugar sand formed and the adjusted percent calcium—1961 season.

Constituents correlated	Correlation coefficient
% sugar sand vs:	
% calcium	.655 **
% potassium	−.557 *
% magnesium	−.339
% manganese	−.292
% phosphorus	−.460 *
ppm iron	−.629 **
ppm copper	−.535 *
ppm boron	−.575 **
ppm molybdenum	−.333
% calcium malate	.620 **
% free acids	−.438
% total malic acid	.597 **
% total organic acids	.608 **
% acids, other than malic	.216
% undetermined material	−.630 **
% calcium (adjusted) vs:	
% potassium	−.428 *
% magnesium	−.496 *
% manganese	−.696 **
% phosphorus	−.678 **
ppm iron	−.818 **
ppm copper	−.903 **
ppm boron	−.908 **
ppm molybdenum	−.283
% total malic acid	.953 **

* Indicates significance at the 5% level.
** Shows significance at the 1% level.

Table 6 also shows the relation between calcium content and several of the other important constituents of sugar sand. The positive relation between calcium content and total malic acid content is highly significant (.953) and further supports the theory concerning the formation of insoluble calcium malate during evaporation. The high negative correlation between calcium content and boron content (−.908) can be partially explained by the established negative effect of calcium on the uptake of boron in plants. Experimental results concerning the highly significant negative correlation coefficients between calcium content and copper (−.903) and iron (−.818) content are still too scanty to permit a general conclusion.

The range of values of every constituent tested during both seasons is quite large (Tables 1 and 2). This is not unusual, since the sirup samples represent different areas within the state. Table 2 compares two runs from the same cooperator, showing only minor differences between the two runs of each producer in the amounts of potassium, magnesium, and manganese. The amounts of magnesium, manganese, and molybdenum in the sugar sand samples are not correlated with the amount of sugar sand deposited, and their presence apparently has no relation to the deposition of sugar sand (Tables 3 and 6).

Nonvolatile organic acids. Fig. 1 gives typical chromatograms showing the location of the organic acids in sugar sand. The *n*-pentanol–5*N* formic acid (1:1 v/v) developing solvent gave the best resolution of the three solvents used in this study. Because of the masking effect of the large amount of malic acid present, three chromatograms were made on each sugar sand sample. Each chromatogram represents the material washed from approximately one-third of a column. The nonvolatile acids were identified by relating the R_f value of each spot on the chromatogram to the R_f

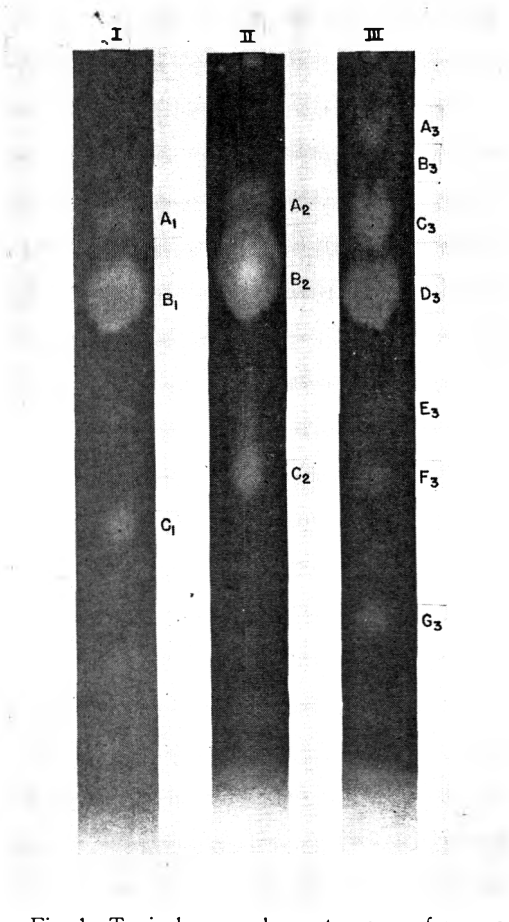

Fig. 1. Typical paper chromatograms of a sugar sand sample, showing the location of the organic acids.

values of the reference compounds. Four of the seven acids present were identified by their R_f values as citric (spots A_1, A_2, and C_3), malic (spots B_1, B_2, and D_3), succinic (spot F_3), and fumaric (spot G_3). The presence of these four acids has been reported by Nelson (1928) and Porter *et al.* (1951).

It is quite evident in Fig. 1 that the concentration of malic acid was considerably higher than that of the other acids. These results, supported by the chemical analysis of the sugar sand shown in Tables 1 and 2, are attributed to the relatively high concentrations of calcium malate in sugar sand.

The presence of other unidentified acids in maple products was reported by Willits (1958), whose results indicated the presence of four unidentified acids in maple sap and sirup and three additional unidentified acids in the sirup.

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A Short-Term Effect of Weather on Malic Acid in Pineapple Fruit[a]

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SUMMARY

The malic acid content of pineapple fruit is quite sensitive to changes in sunlight or conditions favoring water evaporation. By contrast, citric acid is unresponsive to these factors. The inverse relation of malic acid in the fruit and evaporative forces may be related to Crassulacean acid metabolism.

Incidental to a study on composition changes during the development of pineapple fruit conducted by Dr. V. L. Singleton, the writer obtained data on the citric and malic acid contents of the fruit during several months in 1958. These two acids showed very different patterns, with the citric acid rising fairly steadily during the last three months of fruit development and showing little week-to-week fluctuation. Malic acid, in contrast, showed a marked week-to-week fluctuation and no tendency to rise during development of the pineapple fruit. This report shows that the malic acid content in pineapple is unusually responsive to short-term variations in sunlight and other conditions favoring moisture consumption.

EXPERIMENTAL

Two lots of pineapple fruit (*Ananas comosus*, var. Smooth Cayenne) were marked while in the flowering stage. They were selected for uniformity within lots, and the two lots were estimated (and later confirmed at the field-ripe harvest) to be one week apart in stage of development. Ten fruit from each lot were harvested weekly, and the juice from the edible portion was analyzed as duplicate 5-fruit composites.

Citric acid was determined colorimetrically by the acetic anhydride-pyridine reaction, using the procedure of Saffran and Denstedt (1948) as modified by H. Y. Young, of these laboratories. Malic acid was measured by optical rotation of its uranyl complex (Dunbar and Bacon, 1911). The acids were not separated on ion-exchange columns.

DISCUSSION

As shown in Fig. 1, the malic acid was quite irregular throughout the period of fruit development, showing no tendency to rise or fall with stage of fruiting. However, the two lots were remarkably consistent in showing rises and dips in the malic acid level. This indicated that some factor in the environment was influencing the malic acid content of the fruit.

Earlier unpublished work by the writer had shown that the hydroxycinnamic acids

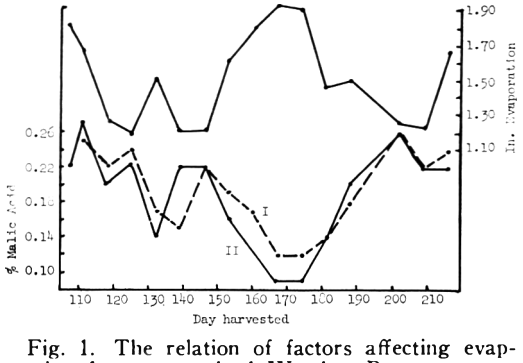

Fig. 1. The relation of factors affecting evaporation from a standard Weather Bureau pan to malic acid content of developing pineapple fruit. The pan evaporation data are for the week ending 7 days prior to fruit harvest. The fruit of lot I were one week behind lot II in stage of development; the fruit were flowering at the first sampling and field-ripe at the final sampling.

[a] Approved by the Director as Technical Paper 289 of the Pineapple Research Institute.

in pineapple stem tissue—and in the stems of crowns of the same fruit used in the present test—varied inversely with soil moisture. However, neither soil moisture nor rainfall patterns correlated with the malic acid level in fruit. Soil and air temperatures also failed to vary in the pattern observed for malic acid.

An excellent inverse correlation was found for fruit malic acid and moisture consumption as measured by evaporation from a standard Weather Bureau pan (Fig. 1). There appeared to be a lag of about a week before the effects of the weather factor showed up in the malic acid content. The correlation appeared to be best when malic acid was related to one week's accumulated evaporation ending 7 days prior to picking the fruit. Sunlight appeared to be the factor of greatest importance; radiant energy (recorded as calories per square centimeter per week) also varied inversely with malic acid in the fruit, roughly paralleling pan evaporation.

It is interesting to note that the malic acid was slower to rise and slower to fall in the less-developed lot of fruit than in the lot one week older. A similar lag was observed in the synthesis and breakdown of citric acid and numerous other components in the juice during development of the younger lot of fruit.

These data emphasize that the two major organic acids of pineapple—citric acid and malic acid—are influenced by quite different factors. Citric acid apparently varies primarily with stage of fruit development. Malic acid, on the other hand, fluctuates in association with weather conditions favoring evaporation of water. The malic acid in the fruit appears to be metabolized during periods of high light intensity and accumulates when sunlight and evapotranspiration are low.

The possibility that these observations are related to "Crassulacean acid metabolism" (Ranson and Thomas, 1960) has been suggested by work of Dr. Paul Ekern, of these laboratories, as well as by earlier observations here that pineapple leaves show marked diurnal variations in malic acid content (Sideris *et al.*, 1948). The Crassulacean-metabolism features of pineapple leaf acidity have been confirmed by more recent work in India (Seshagiri and Suryanarayanamurthy, 1957).

ACKNOWLEDGMENT

I am indebted to V. L. Singleton for the fruit samples, Paul Ekern for weather data, and H. Y. Young and Seichi Shimabukuro for technical assistance.

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RESEARCH NOTE

Non-volatile Organic Acids[a] of the Dwarf Cavendish (Chinese) Variety of Bananas

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The 'Dwarf Cavendish' variety, known locally as "Chinese" banana (Simmonds, 1954), is the most widely grown dessert banana in Hawaii. The plant of this variety is relatively short and is considered to be one of the Cavendish group of bananas described in detail by Simmonds (1959, pp. 53, 79, 105). Although it is known and appreciated for its distinctive flavor in other areas of the world, it has not received the amount of study reported for the Gros Michel.

The organic acid composition of several banana varieties, especially the Gros Michel, has been reported (Steward *et al.* 1960; von Loesecke, 1949). Knowledge of the organic acids in the Chinese banana is needed in evaluation of problems in the handling, storage, and processing of this variety. The following information reports on a preliminary exploration of the non-volatile organic acids in this variety.

Banana ripeness was estimated by reference to color plates (Fruit Dispatch Co., 1956) that score ripeness by eight color changes of the skin ranging from green to full ripe. Used in this investigation were bananas of Nos. 2, 4, 6, and 8 degrees of ripeness—respectively green to yellow ripe.

Alcoholic extracts of banana pulp were analyzed by descending paper partition chromatography, using Whatman No. 1 paper. Extracts had been purified by cation and anion exchange (Porter *et al.*, 1951). Results indicated that malic and citric acids were the predominant acids. Phosphoric acid was identified by the molybdate benzidine reaction. Three additional acid spots were noted that are not acids previously reported as being present for the Gros Michel variety. R_f values are reported in Table 1. Values for the unidentified acids correspond to some of those reported by Steward *et al.* (1960), e.g., value No. 1 to his reported No. 17, and No. 3 (Table 1) to No. 27. The latter could not be verified as succinic acid. Value No. 2 (Table 1) was not considered to be represented among the unidentified acids reported by Steward *et al.*

Banana extracts were fractionated on silicic acid columns (Bulen *et al.*, 1952). Malic

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Table 1. R_f values for unfractionated banana extracts in solvent compared with values from known standards.[a]

	Extract	Malic	Extract	Citric	Extract	Phosphoric	Unknown acid values 1	2	3[b]
No. 2 ripeness	.52	.53	.45	.46	.26	.28	.12	.65	.....
No. 4 ripeness	.52	.53	.45	.46	.26	.28	.12	.65	.....
No. 6 ripeness	.53	.53	.46	.46	.27	.28	.12	.65	.79
No. 8 ripeness	.53	.53	.46	.46	.26	.28	.12	.66	.....

[a] Solvent a: *n*-butanol, formic acid 88%, water (4:1:5).
[b] Spot not detectable in ripeness Nos. 2, 4, 8.

Table 2. R_f values obtained with three solvents after silicic acid fractionation, compared with values from unfractionated known acids.

Sample	Solvents:[a]	Fractionated Malic a	b	c	Malic a	b	c	Fractionated Citric a	b	c	Citric a	b	c
Known mixture		.53	.53	.39	.53	.53	.39	.45	.46	.23	.45	.46	.23
50:50 mixture of known and extract		.52	.54	.39	.52	.54	.39	.44	.48	.24	.44	.47	.24
Extracts of:													
No. 2 ripeness		.55	.53	.39	.54	.53	.39	.47	.48	.24	.47	.47	.23
No. 4 ripeness		.54	.55	.40	.53	.55	.40	.46	.47	.25	.46	.48	.25
No. 6 ripeness		.56	.56	.36	.54	.56	.36	.47	.48	.19	.47	.48	.20
No. 8 ripeness		.53	.56	.35	.53	.58	.35	.47	.48	.20	.47	.48	.20

[a] Solvent *a*: *n*-butanol, formic acid 88%, water (4:1:5).
b: Benzyl alcohol, tertiary butanol, isopropanol, formic acid, water (3:1:1:1:1).
c: Normal propanol, ammonia, water (6:3:1).

and citric acids were separated and identified by chromatographic comparison with pure acid solutions, alone and added to the extracts. It is estimated that these acids may account for as much as 80% of the total acid fraction with the methods used. R_f values obtained after fractionation with three solvents are reported in Table 2.

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Isolation of Gram Quantities of a Rhamnoglucoside of Apigenin from Grapefruit

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(Manuscript received June 12, 1962)

SUMMARY

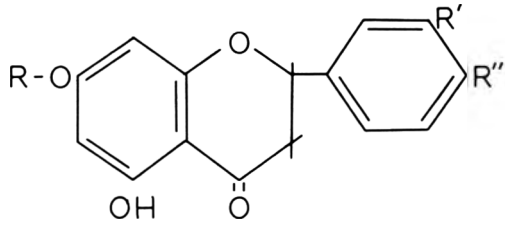
Commercial naringin, usually obtained from grapefruit peel, was suspected to contain a flavone. A method was devised without resorting to column chromatography whereby gram quantities of the suspected flavone were obtained. The crude flavone was first purified by solvate formation. After conversion of the flavanones present to water-soluble flavans by potassium borohydride reduction, the insoluble flavone was obtained. Characterization of the flavone led to the conclusion that it was a 7-rhamnoglucoside of apigenin identical to rhoifolin.

INTRODUCTION

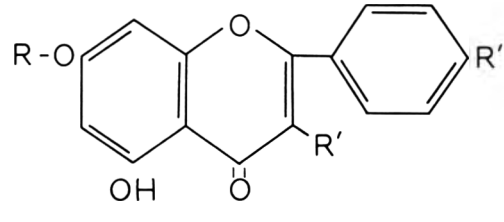
Naringin, one of the bitter constituents of grapefruit (*Citrus paradisi*) has been known for over a hundred years. A comprehensive review on naringin was published by Kesterson and Hendrickson (1953). Horowitz and Gentili (1961) recently found poncirin (7-neohesperidoside of isosakuranetin) to be present in grapefruit.

From the leaves of the Japanese wax tree (*Rhus succedanea*), Hattori and Matsuda (1952) were the first to isolate a flavone closely related to naringin. They called it rhoifolin and established its structure to be the 7-rhamnoglucoside of apigenin. The Japanese hitter orange (*Citrus aurantium* L.) was later found to contain rhoifolin (Hattori *et al.*, 1952). Matsuno (1959) also isolated rhoifolin from *Citrus grandis* Osbeck. From the above findings, and the fact that commercial naringin reacted positive to the borocitric color test for flavones (Wilson, 1939) it was decided to attempt to isolate the flavone, which research is now reported. Dunlap and Wender (1962) recently chromatographically isolated rhoifolin, neohesperidin, poncirin, and kaempferol from commercial naringin.

An additional reason for isolating rhoifolin stems from interest in non-toxic spasmolytic agents. Apigenin, and related aglycones, have been reported to possess significant spasmolytic activity (Janku, 1957; Shibata *et al.*, 1960). The relations of the above flavonoids are illustrated in Fig. 1.



R=NEOHESPERIDOSE •
R'=H, R''=OH (NARINGIN)
R'=H, R''=OCH_3 (PONCIRIN)
R'=OH, R''=OCH_3 (NEOHESPERIDIN)



R=NEOHESPERIDOSE •
R'=H, R''=OH (RHOIFOLIN)
R=H, R'=OH, R''=OH (KAEMPFEROL)

Fig. 1. Relations of several flavonoids.

EXPERIMENTAL

Five hundred and twenty grams of commercial-grade naringin were added to 15 L of 99.6% isopropyl alcohol. The solution was stirred two days, and the crystalline solvate of naringin was removed by filtration. Concentration of the alcohol fraction under vacuum to a volume of 4 L caused a second crop of naringin isopropyl solvate to precipitate. This solution was filtered and the alcohol evaporated. The residue (29 g) was treated as follows: The residue was taken up in water, air dried, and dissolved in methyl ethyl ketone. The crystalline material (3 g) that formed upon stirring was collected by filtration. It was temporarily water soluble, but after standing reprecipitated. The precipitate was collected, dissolved in 250 ml of 0.1*N* sodium hydroxide, and 5 g of potassium borohydride (KBH_4) was added. Acetic acid (0.5*N*) was added slowly. Upon completion of the reduction reaction the flavone precipitated, while the reduced flavanones (the chief contaminates) remained in solution. Bauer *et al.* (1954) reported the reduction of flavanone with sodium borohydride. Horowitz (1957) used sodium borohydride followed by hydrochloric acid to detect flavanones on paper chromatograms. Work in our laboratories indicated that flavones such as diosmin or rhoifolin could be recovered unchanged after treatment with sodium or potassium borohydride. Recrystallizing the flavone several times through methanol yielded 1.08 g of light-yellow, minute, needle-like crystals. Acetylation and methylation of the aglycone indicated the material was a 7-glycoside of apigenin. Paper chromatograms and infrared spectra were identical with authentic rhoifolin kindly supplied by Prof. S. Hattori, of the University of Tokyo. See Table 1 and Fig. 2.

Table 1. Chromatographic comparison of rhoifolins.

Sample	Solvent R_f's × 100 HOH	15% HAc	BAW (4:1:5)
Authentic rhoifolin [a]	15	42	45
Grapefruit rhoifolin	14	43	45

[a] Kindly supplied by S. Hattori.

IDENTIFICATION

Hydrolysis. One gram of the flavone glycoside was dissolved in 100 ml of methyl cellosolve and poured into 1 L of boiling 3% sulfuric acid. After one hour of boiling the mixture was allowed to cool. The precipitate was collected; the yield was 429 mg. The ultraviolet spectra and melting point agreed with those of apigenin. A portion of the liquor from the above hydrolysis was neutralized with $Ba(OH)_2$ to pH 7.0. The $BaSO_4$ was removed and the liquor brought to dryness. Calculations based on sample size and percent sugars in a rhamnoglucoside of apigenin indicated about 211 mg expected. Actual weight was 260 mg. This residue was examined by paper chromatography in *n*-butyl alcohol–acetic acid–water (4:1:5), ethyl acetate–water–pyridine (2:2:1), ethyl acetate–water–acetic acid (3:3:1), and the R_f's agreed with those of glucose and rhamnose. See Table 2.

Acetylation of the Aglycone

The method of Hattori and Matsuda (1952) was used, with modifications. One drop of pyridine was added to 0.1 g of the aglycone dissolved in hot acetic anhydride (2 ml). After refluxing for 1 hr on a water bath, the mixture was cooled and poured into water, and the precipitate col-

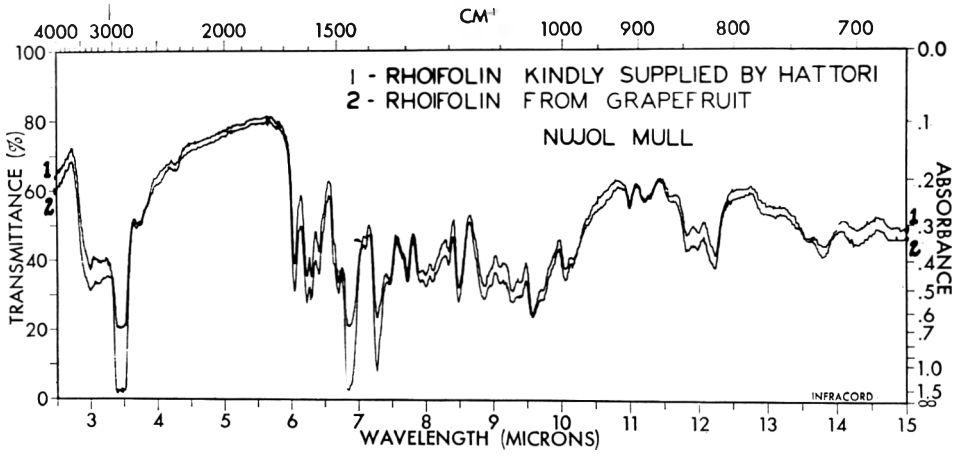


Fig. 2. Comparison of paper chromatograms of rhoifolin.

lected. Recrystallization from methanol resulted in colorless needles with m.p. 179–181°C (literature, 180–181°C) (Hattori and Matsuda, 1952).

Methylation of the Flavone Glycoside Followed by Hydrolysis: Preparation of 5,4'Dimethyl Apigenin

To 0.2 g of the flavone glycoside in 50 ml of acetone were added 1.5 g of potassium carbonate and 1 ml of dimethyl sulfate. The mixture was refluxed for 4 hr on a water bath. Ten ml of water was added, and the acetone distilled. Upon cooling, a solid was collected. Hydrolysis in 3% sulfuric acid resulted in a precipitate that was recrystallized through methanol several times; m.p. 265–267°C (literature m.p., 262–275°C) (Hattori and Matsuda, 1952).

Table 2. Sugars of rhoifolin.

Sample	Solvent Rf's × 100 BAW (4:1:5)	EWP (2:2:1)	EWA (3:3:1)
Glucose	26	48	15
Unknown	25	48	17
Rhamnose	40	61	28
Unknown	40	61	27

Spots detected by method of Gordon *et al.* (1956): periodic acid followed by benzidine.

DISCUSSION

Following this phase of the work, determination of the amounts of rhoifolin present in the whole fruit was undertaken. Fifteen grapefruit (*Citrus paradisi* var. 'Marsh,' white) grown in Rialto, California, were peeled. The juice was extracted by gentle pressure of the cut fruit portion. Peel, rag, and juice fractions were separated. Recovery of the rhoifolin from the peel and rag was accomplished by alkaline extraction followed by neutralization. The precipitates were analyzed using the borocitric color reaction and the following amounts of rhoifolin were estimated: peel, 1 mg/100 g (dry-wt basis); rag, .02 mg/100 g (dry-wt basis).

The juice portion was extracted with methyl ethyl ketone in a liquid-liquid extractor. Because of the complex nature of this extract, no positive statement can yet be made on the amount of rhoifolin in the juice. The extract is now being chromatographed on silicic acid.

CONCLUDING REMARKS

It is interesting to speculate if rhoifolin might be the end product formed from naringin as grapefruit matures. It is a well established fact that grapefruit juice becomes less bitter with maturity. Evidence to date does not indicate that naringin is converted to naringenin or prunin. Therefore, some other route must be the natural one. Since rhoifolin is non-bitter, this may be the substance that accumulates at the expense of the naringin.

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Estimation of Extra-Cellular Starch of Dehydrated Potatoes

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(Manuscript received August 7, 1962)

SUMMARY

Extra-cellular starch is isolated from dehydrated mashed potatoes by rehydration of 0.5-g samples with 100 ml water at 150°F. The suspension is poured over a standard 150-mesh sieve, and the water containing the freed starch gel collected and filtered. Starch so collected on 7-cm hardened and extremely retentive filter paper is stained with iodine, and its reflection density (R_d) and color characteristics measured on a color-difference meter. Transmission values may also be determined. In addition, extra-cellular starch, so collected from 1-g samples, may be dried and weighed. R_d values are a linear function of the amount of starch. The temperature of the sample suspension also has a linear effect on reflection density.

Since added stearates do not interfere with reflection density values, the effects of added emulsifiers on iodine color of the collected starch gel are measurable by color-difference procedures. Color difference also provides a means of characterizing other starch changes, such as retrogradation, induced by processing treatments.

An insignificant number of the smallest intact cells pass through the screen to be collected on the filter paper. The intact, separated cells of cooked potato range from less than 40 to nearly 400 μ in diameter. They average about 180 μ, and over 90% have diameters between 100 and 250 μ.

INTRODUCTION

Sticky or gummy texture in dehydrated mashed potatoes results from excessive amounts of gelled starch extruded from ruptured cells (Harrington *et al.*, 1960; Mullins *et al.*, 1955; Reeve, 1954). Percent of cells ruptured thus provides a general index for textural quality (Reeve and Notter, 1959). The degree to which individual cells are damaged may vary, however. Microscopic examinations of some products reveal many of the broken cells to be so completely torn that most of their gelled starch content is liberated. Other products with a similar incidence of ruptured cells show only small amounts of extruded gelled starch.

Addition of whole starch, which is gelled during reconstitution, is known to impair the reconstitution texture of potato granules, as measured by the "drop test" (Harrington *et al.*, 1960). Colorimetric measure of the amylose readily extracted with water at 150°F provides a good texture index when products of similar manufacture are compared (Mullins *et al.*, 1955). With this test, the "Blue Value Index," it may be assumed that the soluble starch is more readily extracted from the extra-cellular gelled starch than from the starch gel present in the intact cells. Ready extractability of the amylose, however, is influenced by the degree to which the starch may have been retrograded during and after processing (Kerr, 1950; Potter *et al.*, 1959). In addition, use of emulsifiers in the manufacture of both potato flakes and granules is known to reduce the obtainable "blue value."

Bourne *et al.* (1960) have shown that sucrose monostearate complexes readily with soluble starch. The complex formed inhibits its iodine absorption in the helix of the molecule. Thus, a product could yield a low "blue value" but contain sufficient free

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gelled starch to render the reconstituted product sticky. Moreover, additional cell rupturing may occur upon reconstitution. Further knowledge of the extra-cellular starch, *per se*, seems desirable.

There have been no methods described that provide for isolation and observations on the total extra-cellular starch. The new method developed in the present studies permits collection of the extra-cellular starch of dehydrated potato products after it has been washed free from the product by an excess of hot water. This starch, collected on filter papers by vacuum filtration, may be weighed —or stained with iodine and estimated colorimetrically by either reflectance or transmission procedures. In addition, the soluble starch of the filtrate may be measured colorimetrically. Reflectance procedures permit color-difference measurements of the starch-iodine complex, which reveal the influence of emulsifiers and other processing treatments on the iodine-staining characteristics of the freed starch.

METHODS

A carefully weighed 0.5-g sample of dehydrated potato granules is stirred into 100 ml water at 150°F and stirred intermittently for 3 min. After 3 min, the sample is stirred vigorously about 30 sec to separate cell clusters, and then poured rapidly and evenly over a standard 150-mesh screen (100-μ openings) prewet with hot water and placed over a collecting pan. The collected liquid containing the gelled starch is filtered through a Buchner funnel, using mild suction and filter paper of 7 cm diameter, "hardened and extremely retentive," and alkali-resistant (such as S & S No. 576). Washing of settled gelled starch from the pan is unnecessary if the liquid in the pan is vigorously swirled just before transfer.

The filter papers containing the trapped starch gel are stored temporarily in closed Petri dishes until tested; forceps are used for handling all filter paper. Although the papers could be dried and stored for longer periods, it is preferable to standardize the time for shorter periods and to test the samples for the reflection densities of their stained, collected starches within 1–2 hr of collection. The filtrates can be saved and tested for "blue value" (Mullins *et al.*, 1955) if desired.

In this study, some samples were also suspended in water at various other temperatures up to 212°F in order to follow the influence of temperature on the amount of starch obtained and the iodine color. Both experimental and commercial granules were used in these studies. Effects of stearates on transmission and reflectance data were investigated by addition of either glycerol monostearate or sodium stearate to the products at the time of suspension; they were also added to prepared potato starch samples. Appropriate amounts of 1% aqueous emulsions of the stearates were added by pipette to the heated water in which the samples were suspended.

Reflectance procedures. The staining reagent consists of 2 ml of a stock preparation of 7.5 g each of I_2 and KI in 250 ml distilled water, further diluted with 248 ml distilled water. A preparation of 0.02N KI_3 may be used, but this was found to be less satisfactory for very small amounts of collected starch gel, particularly in color analyses of the starch-iodine color complex, because of the deeper iodine color in the control filter paper.

About 30 ml of the iodine solution is placed in a Petri dish, and the filter paper, starch-side up, is carefully submerged for a standard time such as 30 or 60 sec. The paper is then placed starch-side down on a clean 3.25×4-in. lantern-slide cover glass and a second cover glass added, with care to avoid trapping excess air bubbles. The control filter paper is handled in like manner. This sandwich is then placed starch-side down under the standard opaque white plate of a Gardner automatic color-difference meter, the light intensity having been adjusted according to the standard plate. The reflectance density (R_d) and the A and B color values are then determined within 1 or 2 min of removal of the test paper from the iodine solution. Percent reflectance is taken directly as an index, or the value is converted to density simply by calculation of the log c/t, where c is percent reflectance of the control filter paper and t is that of the test sample. The diluted iodine solution should be replaced after every third or fourth determination.

Transmission colorimetry. Alternately, transmission colorimetry may be used. The suspension of collected starch gel can be measured directly by the "blue value" method of Mullins *et al.* (1955), or filtered and the "blue value" of the filtrate determined. If measured directly, 49 ml of thoroughly mixed suspension is removed (during stirring) and 1 ml of the 0.02N KI_3 solution added. A reading is made immediately with a photoelectric colorimeter, such as the Klett-Summerson, using a red filter in the range of 640–700 mμ. For measurement on the filtrate, 5 ml of the filtrate is diluted to 49 ml with water before addition of the KI_3 solution. In either case, the control consists of 49 ml water plus 1 ml of the KI_3 solution.

Weight of extra-cellular starch. When the extra-cellular starch is to be weighed, it is expedient to use 1-g samples of product. The extra-cellular starch is collected on pre-weighed filter papers. The filter-paper samples, placed in loosely closed, labeled Petri dishes, are dried 24–48 hr at about 45°C. They are then allowed to equilibrate with the air at room temperature and humidity (3 to 4 hr) before being weighed on an analytical balance. A control filter paper, treated with water only at the temperature of sample suspension, is used to provide correction for weight change in the filter paper due to humidity. Storage of filter paper samples for 24–48 hr over a saturated solution of NaBr (to provide an atmosphere of 50% relative humidity) after drying did not significantly alter final corrected weights. Similar storage over P_2O_5 was found to be impractical when more than one or two samples were prepared at a time, because the rapid weight change due to moisture uptake dictates use of a constant-low-humidity room.

OBSERVATIONS AND RESULTS

Retention of intact cells. Retention of intact cells and passage of the extra-cellular starch are essential. Efficiency of the 150-mesh screen for such separation was verified by numerous microscopic examinations of filter papers and also by size analyses of the rehydrated potato cells suspended in water in counting chamber slides such as those used for ruptured cell counts. Cell diameters were measured with a calibrated eyepiece micrometer, and size distributions based on 20-μ intervals were determined on samples of 1,000 cells each. Typical data are listed in Table 1.

About 4% of the rehydrated cells had diameters less than 100 μ, and about 2/3 of these belonged in the range of 80–100 μ. Calculated as spheres, these small cells made up less than 1% of the total volume of intact cells. Material retained on the screen and resuspended showed size frequency distributions virtually identical with those of original material. Frequency counts of cells per 20 microscopic fields for suspensions of equal volume were likewise the same before and after screening.

Microscopic examinations of samples on filter papers prepared from potato granules of different manufacture and degree of cell damage revealed no differences in presence of small intact cells; in all cases a few small intact cells and a few of those ruptured had passed the screen. In addition, nearly all samples showed a very few badly torn cells. Fig. 1 is from a sample of medium to low extra-cellular starch having about 4% cell rupture. The linear dimensions of the gelled starch particles on the paper average much larger than those of the small intact cells. Cup-like pockets of cell walls of badly torn cells also contain some gelled starch. Particles of starch gel on the papers ranged up to 25 or more per sq mm, depending on the amount of extra-cellular starch in the original sample. Counts of the small intact cells averaged less than 1 per sq mm, and only about 10% of these had diameters of 100 μ. It seems reasonable that less than 1% of the assumed available small cells of the original sample had passed the screen.

Microscopic examinations of the reverse sides of the filter papers revealed no starch particles pulled through the paper. Repeated cold-water washings of material retained on the screen, when filtered and stained, revealed only very minute amounts of additional starch gel, and gave reflectance values very close to those for the control. It is evident that the screening operation is effective in isolating the extra-cellular starch.

Linearity of the gelled starch index. Known amounts of gelled potato starch were prepared from suspensions of 500 mg of an extracted starch in 500 ml of water at 150° and at 180°F. After the suspensions cooled to room temperature, appropriate aliquots were pipetted, during thorough mixing, and diluted to provide equivalents of original weights of 1, 1.5, 2, 2.5, 3, 4, and 5 mg each in 100 ml of water at 150°F. These diluted suspensions were then filtered, and reflectance values obtained. Replicate samples were prepared for determinations of weights of collectable starch gel.

Fig. 2 illustrates the linear relation between reflection density and starch concentration, and also demonstrates removal of more amylose from

Table 1. Size distribution of intact cells of rehydrated potato granule.

Maximum diameter (μ)	Cumulative % below
40	0.1
60	0.4
80	1.5
100	4.0
120	10.0
140	18.0
160	32.0
180	48.0
200	66.0
220	80.0
240	90.0
260	94.5
280	96.5
300	98.5
320	99.5
340	99.9
380	100.0

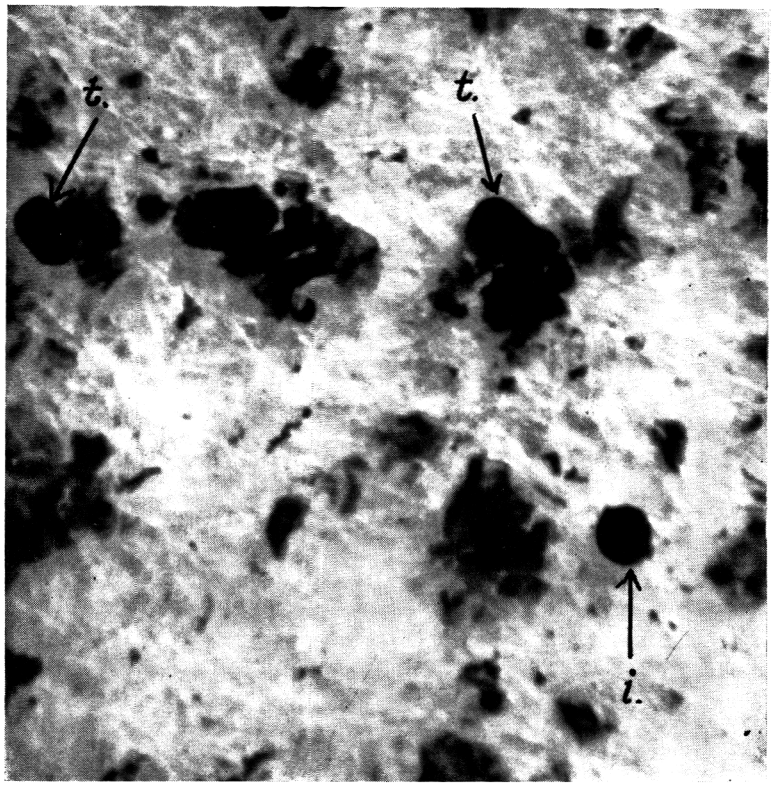

Fig. 1. Photomicrograph of an area of a filter-paper sample showing collected starch gel stained with iodine, and a small intact cell (i) and 2 badly torn cells (t) (× 100).

the gelled starch with the higher temperature of preparation. The greater increases in R_d for preparations gelled at 150° indicate progressively greater amounts of total starch gel collected than for those gelled at 180°F. The starch grains were less swollen by the 150° treatment than by gelation at 180°, and lost less of the soluble amylose fraction. Tests of the filtrates for soluble starch gave "blue values" too low to show significant trends for most of these samples. However, the weights of collected starch on filter papers for replicate samples were uniformly 15–20% less than amounts calculated from original suspensions for those gelled at 150°, and 20–25% less for those gelled at 180°F. Other samples prepared with prolonged heating and at higher temperatures, as in the cooking of potatoes, showed proportionately less increase in reflection density with increased starch, but linear relation was maintained.

In another experiment, 0.5-g samples of an experimental potato granule were suspended in 100 ml water at 140, 150, 160, 170 and 180°F. Relation between temperature of suspension and reflectance density for the collected starch gels of these samples is illustrated in Fig. 3. Microscopic counts of ruptured cells failed to reveal any significant increase in percent of cells ruptured with increased temperature for this particular granule. However, the amount of starch exuded from ruptured cells increased visibly with increased temperature treatment. That is, the starch gel swelled more; larger amounts covering larger areas could be observed both on filter papers and in suspensions prepared for cell counts.

Cell rupture and reconstitution temperature. The degree of cell rupture increased with increase in temperature of reconstitution of some products, notably those that tended to be sticky when prepared with boiling liquid. Several of these are compared in Table 2 as to cellular damage, reflectance density, and reconstitution quality. A general relation of high reflectance values to stickiness is evident.

As should be expected, products of different manufacture vary in degree of cell rupture and in effects produced by different conditions of reconstitution. A and B in Table 2 are experimentally produced granules from the same source material and processed similarly except for treatment to induce more starch retrogradation in B than in A. Thus, when rehydrated at 212°F, B shows less cell damage, lower reflectance density, and better texture than A. Sample C is an experimental granule in which faulty processing caused excessive cell damage. The results for D show

Table 2. Relation of cell rupture to reflectance density of stained starch, reconstitution conditions, and texture.

Sample	Preparation		% of cells ruptured	Reflectance density (log c/t)	Texture with culinary reconstitution	
					170°	212°
A	0.5 g suspended directly,	150°F	3.2	0.31	mealy	sl. sticky
		212°F	6.7	0.92		
B	0.5 g	150°F	3.1	0.14	grainy	mealy
		212°F	4.5	0.43		
C	0.5 g	150°F	15.0	1.20	sticky	gummy
D	0.5 g	150°F	6.4	0.52	creamy	gummy
		212°F	8.5	0.95		
D	Culinary reconstitution at 170°F, then 2.5 g suspended at 150°F for collection of free starch		6.4	0.92	creamy	
D	Culinary reconstitution at 212°F, then 2.5 g suspended at 150°F for collection of free starch		10.5	1.05		gummy

Table 3. Cell rupture and starch characteristics of samples suspended at 150° and 200°F.

Sample and temp. of suspension	% of cells ruptured	Collected starch (mg)	Reflection density (log c/t)	Color difference	
				A+ (red)	*B*− (blue)
A—150°	3.0	6.0	0.29	5.0	13.3
200°	6.0	7.5	0.60	8.5	25.2
B—150°	12.0	11.5	0.53	3.0	26.3
200°	25.0	18.5	0.98	3.2	44.7
C—150°	4.5	3.5	0.21	3.2	11.4
200°	7.0	5.5	0.55	6.0	27.9
D—150°	4.0	5.0	0.23	5.1	11.7
200°	7.0	9.5	0.64	9.3	26.6
E—150°	18.0	9.0	0.45	3.6	21.6
200°	28.0	11.5	0.77	3.7	36.4
F—150°	7.0	8.5	0.40	4.9	19.3
200°	12.0	10.0	0.68	6.5	34.7
G—150°	3.0	6.0	0.31	3.5	17.8
200°	4.5	7.5	0.57	5.0	28.2
H—150°	3.0	3.0	0.22	2.4	12.2
200°	4.5	4.0	0.41	3.7	23.2
I—150°	4.5	5.5	0.25	2.6	15.2
200°	6.0	8.0	0.56	4.0	28.7
J—150°	3.0	2.5	0.18	2.2	12.5
200°	5.0	4.5	0.47	4.2	24.5
K—150°	1.5	1.5	0.13	1.2	8.6
200°	2.5	2.0	0.39	2.8	18.7

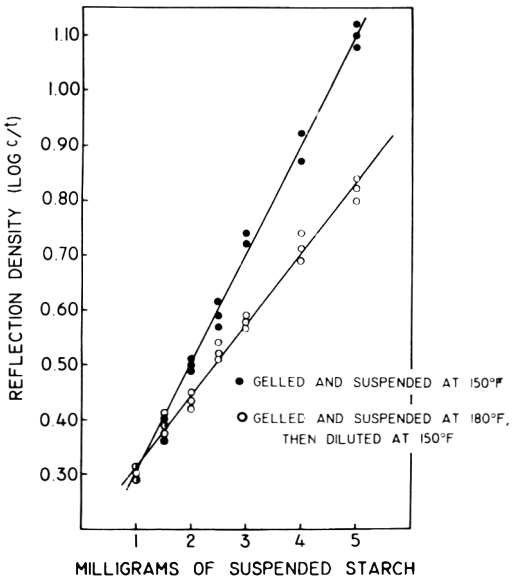

Fig. 2. Reflection density (R_d) obtained with starch preparations of known concentration.

excessive cell damage upon culinary reconstitution at 212°, and consequent texture impairment.

Fig. 4 depicts the linear relation between amount of extra-cellular starch and reflection density for several granules, each prepared at suspension temperatures of 150 and 200°F. Two of these (triangles) are of commercial source and processed with added emulsifier; the rest (circles) are experimentally produced granules, only one of which contains added emulsifier (R_d 0.23 and 0.64 for 150 and 200°F, resp.). In general granules yielding 7 or more mg of starch per g of product suspended at 150°F were either sticky or gummy upon culinary reconstitution at 212°F. However, no sharp line can be drawn for a textural quality index, because of the influence of starch-complexing emulsifiers and other factors influencing the properties of the extra-cellular starch.

Further data for the products in Fig. 4 are listed in Table 3, including degree of cell rupture and color difference in their iodine-stained starches. The color differences here were obtained by correction for the background of iodine color in the filter papers; that is, the values of the iodine-treated control filter papers were subtracted from those registered by the test sample. Usually, the R_d color values of the controls registered in the $A-$ (green) and $B+$ (yellow), whereas the test samples register in the $B-$ (blue) and with a small value in either $A-$ (green) or $A+$ (red). To correct for the background iodine color, the blue values of test samples are appropriately increased and all the A values are converted to the red or $A+$. Graphically, this correction transfers the test values to the purple quadrant, with variable proportions of blueness and redness (see Fig. 5).

As shown in Table 3, the corrected blue color component increases with temperature of sample

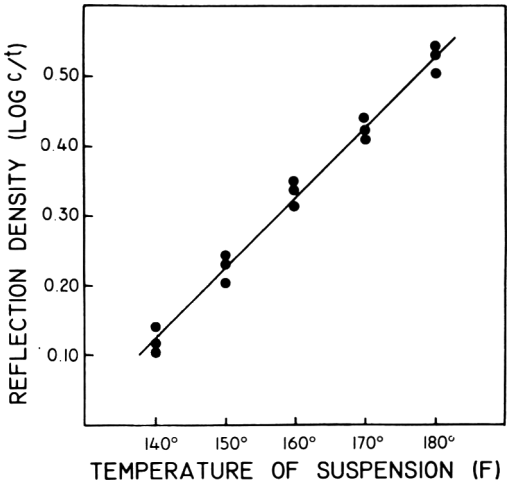

Fig. 3. Influence of the temperature of suspension on the reflection density (R_d) of isolated and stained starch from dehydrated potato granules.

Table 4. Influence of added stearates on transmission and reflectance of the stained starch.

Sample (diluted at 150°F)	% of cells ruptured	Transmission values: Original suspension	Transmission values: Filtrate	Dilute filtrate (BVI)[a]	Reflectance density (log c/t)
A collected starch; granule	3.0	36	25	6	0.29
B collected starch; flake	5.5	335	242	29	0.35
C collected starch; granule	12.0	565	440	56	0.70
C collected starch, plus 2.5 mg glycerol monostearate	12.0	162	90	11	0.73
C collected starch, plus 2.5 mg sodium stearate	12.0	420	370	45	0.71

[a] Blue-value index.

Table 5. Influence of added stearates on reflectance and color characteristics of the stained starch.

Sample and preparation	Color difference		Reflectance density (log c/t)
	$A+$ (red)	$B-$ (blue)	
2.5 mg starch gelled at 180°F, then suspended at 150°F	4.3	29.7	0.54
As above, plus 2.5 mg glycerol monostearate when suspended at 150°F	10.5	27.8	0.57
As above, but with sodium stearate	7.7	30.8	0.55
Granule E (Table 3) suspended at 150°F	3.6	21.6	0.45
Granule E, as above, plus 2.5 mg glycerol monostearate when suspended	8.3	20.3	0.43
Granule E, as above but with sodium stearate	4.0	23.6	0.45
Granule K (Table 3) suspended at 150°F	1.2	8.6	0.13
Granule K, as above, plus 2.5 mg glycerol monostearate when suspended	2.7	8.1	0.12
Granule K, as above, but with sodium stearate	1.9	9.1	0.14

suspension and in general proportion with increased cell rupture and amount of collected starch for each product. The higher red values at a given temperature of sample suspension for 4 of the samples indicate a readily visible shift from the more typical blue, as produced by iodine with amylose, toward the red-purple staining of potato amylopectin. Samples A and D stained distinctly red-purple, C and F were slightly less red-purple than A and D, and all other samples appeared blue-purple to the eye. Such a shift toward red-purple may result from starch retrogradation or from the inhibition of starch-complexing additives on the iodine-binding capacity of the starch.

Influence of added stearates. Stearates were added in powder form to the products prior to weighing and suspension of 0.5-g samples. Transmission values both for the original suspension of extra-cellular starch and for the soluble starch of the filtrate were reduced by these starch-complexing agents (Table 4). However, the reflectance density of the collected starch gel was not significantly altered. Addition of the emulsified stearate to the water in which 0.5-g samples were then suspended did not seem to cause the reflectance density values to differ appreciably from effects obtained with added dry stearate.

Iodine-starch color is altered by the added stearates, and this alteration may be pronounced under certain conditions (Table 5); the value for the red color component increased markedly with added stearate. With the prepared stearate-starch complexes, an increase of 2 units in the red component could usually be detected as a visible shift toward red-purple, but when only 2 or 3 mg of extra-cellular starch from a granule constituted the sample, the red color change of only one recorded unit was readily visible. No significant changes in R_d due to added stearate were obtained with any of the levels of starch tested here, despite the fact that a color filter is not used for reflectance measurements. All color difference values obtained (as in both Tables 3 and 5) fall within the blue-purple to red-purple visible difference (see Fig. 5).

The results agree with those of Bourne *et al.* (1960), who reported that sucrose monostearate forms insoluble complexes with potato starch. The insoluble complex competitively inhibits reaction of starch with iodine, reducing color intensity in the case of transmission measurements and altering the color in the case of reflectance.

CONCLUSIONS

To the author's knowledge, direct measurement of the extra-cellular starch of dehydrated potato products has not been previously reported. Isolation of the extra-cel-

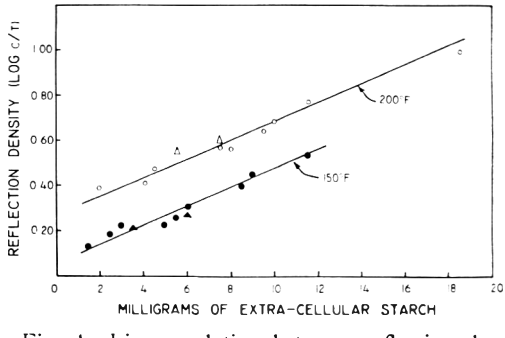

Fig. 4. Linear relation between reflection density (R_d) and amount of collected extra-cellular starch of different dehydrated potato samples after suspension at 150 and 200°F. ○, experimental samples; △, commercial granules.

lular starch gel from these products, as accomplished here, is efficient. Sample preparation is simple and rapid. Relation between degree of cell damage, amount of extra-cellular starch, and textural qualities of the products can be more completely documented than is possible with the more indirect testing methods currently in use.

Reflectance measurement of the stained starch is reliable and sensitive; the values obtained show close linear relation to starch concentration at critical levels and are not adversely influenced by added emulsifiers such as stearates. The deviation of R_d readings for replicate samples, as in Figs. 2 and 3, averages less than 0.025. This is well below the change in R_d value obtained with a difference of 0.5 mg in amount of collected starch gel. The method can be augmented by sensitive analyses of color difference in the starch-iodine complex and by colorimetric transmission measurements. Although no single analytical procedure for establishing a texture quality index has the practical value of simple organoleptic appraisal, these new procedures permit a more complete understanding of conditions that impair reconstitution qualities. They may be applied either to routine tests or to more detailed investigation of starch changes in processed potatoes.

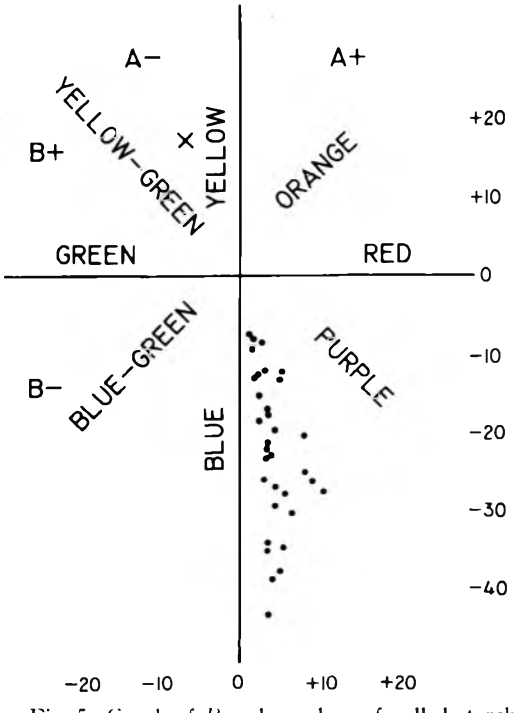

Fig. 5. Graph of R_d color values of gelled starch preparations. X = the color value of a control filter paper soaked in the iodine solution.

Suspension of larger samples and use of filter-paper discs larger than those used here may be desirable when weight of collected starch gel is to be determined. Practical limits are imposed by the area of the screen, however. In general, the efficiency of separation of the extra-cellular starch from the product progressively decreases with use of granule samples exceeding 2 g. Repeated washings of the residue on the screen also result in an increased passage of intact cells smaller than 100 μ in diameter. In addition, filter-paper discs over 7 cm in diameter are awkward to use with the color-difference meter.

The fact that added glycerol monostearate does not interfere with reflection density values requires further interpretation. The readily soluble amylose available to complex with the stearate is precipitated, and iodine absorption in the helix of the amylose molecule is inhibited. The amylose remaining in the gel, however, is free to complex with iodine. Bourne *et al.* found that sucrose monostearate precipitated 78% of the amylose and only 11% of the amylopectin of potato starch.

NOTE

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

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Autoxidation of Beef and Tuna Oxymyoglobins[a]

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SUMMARY

Rates of autoxidation of beef and tuna oxymyoglobins have been determined at pH 5.9 with various buffer concentrations (0.1, 0.2, and 0.6M) and temperatures ranging from 0 to 40°C. The autoxidation is first-order with respect to unoxidized myoglobin under all conditions used. The rates of oxidation of tuna oxymyoglobin were independent of buffer concentration, whereas those of beef myoglobin decreased with increasing buffer concentration. Tuna oxymyoglobin was oxidized more slowly than beef at 0 to 10°C at all buffer concentrations, and at higher temperatures and low buffer concentration; at high temperatures and high buffer concentration, tuna oxymyoglobin was oxidized more rapidly than beef. Oxidation of a crude preparation of beef oxymyoglobin was slower than that of purified myoglobin.

INTRODUCTION

Proper color of meat and fish products is a quality attribute of great importance. In fresh meats and some fish, conversion of the bright-red pigment, oxymyoglobin (MbO_2), to brown-colored metmyoglobin (Mb^+) is the major discoloration problem (Watts, 1954; Brown and Tappel, 1958). This reaction will also be of considerable importance physiologically if myoglobin is susceptible to autoxidation *in vivo*, since Mb^+ cannot be oxygenated.

Snyder and Ayres (1961) recently reported on studies of the rate of autoxidation in air at various temperatures of crystallized beef muscle myoglobin. A discussion of the general problem is included in their paper. They found the autoxidation of beef muscle myoglobin to be essentially the same as that reported by George and Stratmann (1952 a, b) for horse heart myoglobin. In another recent paper, Matsuura *et al.* (1962) described autoxidation rates of myoglobins from fish, sei whale, and horse.

We have been interested in aspects of the comparative biochemistry of myoglobins (Brown *et al.*, 1962) and were particularly interested in determining if the presence of a free sulfhydryl group, apparently unique to fish myoglobins (Brown *et al.*, 1961, 1962), would affect the autoxidation rate. The present study involved comparison at different temperatures of autoxidation rates of highly purified beef and yellowfin tuna myoglobins.

EXPERIMENTAL

Materials. Myoglobins were prepared from aqueous extracts of lean beef and yellowfin tuna (*Neothunnus macropterus*) red muscle by repeated ammonium sulfate fractionation and/or chromatography on diethylaminoethyl cellulose columns as described previously (Brown, 1961). The final preparations of purified myoglobins migrated as single bands during zone electrophoresis on paper or cellulose acetate strips. For the "crude" beef myoglobin preparation, muscle was extracted with water and the extract was dialyzed against several changes of water and finally centrifuged. The resulting extract was clear; no problems with precipitation were encountered. Stock solutions of all preparations were stored frozen; aliquots for day-to-day use were kept at about 5°C.

Methods. A volume of myoglobin solution containing 4.0 mg was diluted to 5.0 ml with phosphate buffer (0.1, 0.2, or 0.6*M*) in a volumetric flask. The final concentration of 0.8 mg per ml is approximately $4.7\times10^{-5}M$. The solution was then reduced by the addition of 2.0 mg of sodium hydrosulfite, transferred to a 16×150-mm test tube,

[a] This investigation was supported in part by a research grant, RG-9899, from the National Institutes of Health, U. S. Public Health Service.

and oxygenated by bubbling a stream of oxygen through it for 1 min. The autoxidation was followed by measuring the decrease in absorbancy at 580 mμ with a Cary Model 11 recording spectrometer; a 1-cm cell was used. For the absorbancy readings the spectrum was recorded from 680 to 480 mμ; thus, the completeness of conversion to the oxygenated form at the outset could be determined, as also could eventual conversion to Mb^+.

For the maintenance of constant temperature during the experiments the tubes were immersed in a water bath controlled to within ±0.5°C for temperatures of 20°C or higher, or controlled to within ±0.3°C for the 10° and 0°C series where a refrigerated bath was used. Absorbancy readings were taken at intervals of 15 or 30 min at the higher temperatures and 1 hr or more at lower temperatures. All readings were made at room temperature, with the samples held at higher temperatures being brought quickly to about 20°C in an ice bath; those held at lower temperatures were warmed in a room-temperature water bath. Runs were continued from 1 hr for the 40° series up to 50 hr for the 0° experiments, and to 96 hours for crude beef myoglobin at 10°. Finally, a few milligrams of potassium ferricyanide were added to obtain complete conversion to the oxidized form.

Taking the initial absorbancy reading at 580 mμ as 100% MbO_2 and the final reading of the oxidized form as 0% MbO_2 (100% Mb^+), a plot of log % MbO_2 vs. time was made for each run. Rate constants were obtained from the slope of the straight-line plots thus obtained.

RESULTS AND DISCUSSION

Among the factors influencing rate of oxidation are: 1) the amount of sodium hydrosulfite used to reduce the myoglobins in the preparation of oxymyoglobin; 2) pH; 3) buffer concentration; and 4) temperature (Snyder and Ayres, 1961; Matsuura *et al.*, 1962). All of our determinations were done on oxymyoglobin reduced with 0.04% sodium hydrosulfite, an amount below the level at which it affects the oxidation rate (Snyder and Ayres, 1961). All determinations were done at a constant pH of 5.9. Since the tuna myoglobins start to be denatured at pH values of around 5.5 we did not wish to use a pH lower than 5.9 in order to allow a margin of safety. Buffer concentration was varied (0.1, 0.2, and 0.6*M*) and the temperatures were 0, 10, 20, 30, 37, and 40°C.

It was found that the autoxidation of purified beef and tuna myoglobins was first-order with respect to unoxidized myoglobin (plots of log % MbO_2 vs. time yield straight lines) at all buffer concentrations and at all temperatures. Our data on beef myoglobin is similar to data obtained by Snyder and Ayres (1961) although actual rate constants, under the same conditions of buffer concentration and temperature, were generally lower in our experiments. We assume that this difference is due to differences in pH (5.9 vs. 5.7) since Matsuura *et al.* (1962) have indicated that rates of oxidation for both fish and mammalian myoglobins increase with decreasing pH. A number of runs at the lower temperatures were continued for prolonged periods, that is, as long as 96 hours; the rates of oxidation of the purified preparations were remarkably constant over the entire oxidation period. Data are shown in Table 1.

Table 1. Rate constants (hr^{-1}) for autoxidation of myoglobins.

°C	Myoglobin	Buffer concentration 0.1*M*	0.2*M*	0.6*M*
0°	Tuna	0.024	0.005	0.002
		.....	0.007	0.004
	Beef	0.054	0.008	0.004
		.....	0.009	.....
10°	Tuna	0.025	0.012	0.009
	Beef	0.161	0.016	0.009
	Crude beef	.....	0.004	0.003
		.....	.....	0.003
20°	Tuna	0.043	0.054	0.048
	Beef	0.357	0.041	0.034
	Crude beef	.....	0.024	0.027
30°	Tuna	0.148	0.175	0.152
	Beef	0.555	0.170	0.093
		0.575	.....	.....
		0.515	.....	.....
	Crude beef	.....	0.053	0.056
37°	Tuna	.....	0.360	0.383
	Beef	.....	0.296	0.194
	Crude beef	.....	0.119	0.112
40°	Tuna	0.681	0.441	0.439
	Beef	1.60	0.655	0.257
		.....	0.577	.....
	Crude beef	.....	0.168	0.124

The effects of buffer concentration were opposite to those reported by Matsuura *et al.* (1962), who found oxidation rates to increase with increase of buffer concentration from 0.2 to 0.6*M*. In our experiments the rates of oxidation of tuna myoglobin were, for the most part, independent of buffer concentration, whereas those of beef myoglobin always decreased with increasing buffer concentration. No explanation is evident.

The tuna myoglobin was oxidized more slowly than beef at low temperatures (0° and 10°) at all buffer concentrations; it was also oxidized more slowly than beef at higher temperatures when the buffer concentration was low, but at higher temperatures and higher buffer concentration the tuna myoglobin was oxidized at rates considerably more rapid that those with beef. Snyder and Ayres (1961) suggested that the best procedure for maintaining the bright-red color of fresh meats would be to keep storage temperatures as low above freezing as possible. The fact that the rates of oxidation of tuna oxymyoglobin show pronounced increases with temperature (i.e. Q_{10} values range from 1.0 between 0 and 10°, to 4.6, between 30 and 40°) indicates that maintaining low temperatures may be of even greater importance for fish products that depend on oxymyoglobin for their fresh appearance.

Activation energies were calculated (Table 2) from oxidation rate measurements at 0, 10, 20, 30, 37, and 40° for both the myoglobins at three buffer concentrations. Our values for beef myoglobin are somewhat lower than those of Snyder and Ayres (1961); again, the only apparent difference in experimental procedure is the difference in pH. Energies of activation of beef myoglobin were more dependent on buffer molarity than were those of tuna.

A number of determinations were performed with a crude beef myoglobin extract; this extract contained seven proteins identifiable by zone electrophoresis on cellulose acetate strips; myoglobin was the major component. Rates of oxidation of the myoglobin were substantially less in this material than in purified beef myoglobin, suggesting that the other constituents of the extract were exerting a protective effect. The oxidation of the crude material followed first-order kinetics for about 1 hour at 40°, and then increased sharply. This effect might be due to loss of protective sulfhydryl groups in some of the other proteins. That such protection is due to proteins is suggested also by the fact that the crude extract had been dialyzed against several changes of water. By implication, therefore, highly purified model systems likely give oxidation rates higher than would be observed in a meat product, assuming no microbial contribution.

Whether the free sulfhydryl of tuna myoglobins has any protective effect is not clear although it may be involved at lower temperatures, at which the rates of oxidation of tuna myoglobin were substantially lower than those of beef. If this is the case, at higher temperatures the sulfhydryl group may be oxidized, the protective effect lost, and the relatively greater rate of oxidation of tuna MbO_2 follows.

Table 2. Activation energies, calories/mole, for autoxidation of myoglobins.

Myoglobin	Buffer concentration		
	0.1*M*	0.2*M*	0.6*M*
Yellowfin tuna	19,900	18,600	21,900
Beef	13,900	20,600	18,000

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Effect of Freezing on Autoxidation of Oxymyoglobin Solutions[a]

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SUMMARY

Rates of oxidation of solutions of highly purified and "crude" tuna and beef oxymyoglobins were measured at 0, −5, −10, −15, and −18°C. The results show that the oxidation rate decreases with lower temperatures until solutions actually solidify; at that time the autoxidation rate increases sharply. There are variations in rates of oxidation between tuna and beef myoglobins, as well as between myoglobin solutions at different buffer concentrations; however, these are secondary to the changes noted due to freezing.

INTRODUCTION

It has been suggested that freezing of meats *per se* is without particular effect on color quality (Weir, 1960). However, it has been shown that temperature and light have marked effects on the stability of color in packaged frozen retail beef cuts, where the primary cause of discoloration is formation of metmyoglobin (Mb^+) (Townsend and Bratzler, 1958). Lane and Bratzler (1962) found that the formation of Mb^+ in frozen-meat water extracts was similar to the pattern seen in frozen steaks, and was significantly increased by exposure to fluorescent lights and by the presence of magnesium and iron chlorides. The primary change in color of fish tissue at 0° is due to formation of Mb^+ (Brown *et al.*, 1957). In most fish, the oxymyoglobin (MbO_2) found along the lateral line changes from bright red to the dull brown of Mb^+ during frozen storage; the change may be sufficient to make the product unsalable (Stansby, 1956).

There have also been tentative suggestions that under certain conditions, color deterioration due to Mb^+ formation was more pronounced in frozen meats and fish than in similar products maintained at low temperatures above freezing. Thus the color of a few samples of beef stored below 0°F was found to be inferior to that of samples stored at 32–34°F (Ball, 1959). Sana *et al.* (1959) found less Mb^+ formation in tuna meat in polyethylene packages at 0° than at −5°C.

Several recent papers have presented data on the autoxidation of various myoglobins at 0°C or higher (Brown and Dolev, 1962; Snyder and Ayres, 1961; Matsuura *et al.*, 1962). In the present work the autoxidation of highly purified beef and tuna myoglobins was studied in solution at temperatures below freezing to determine what, if any, effect the physical change of ice formation would have on the oxidation reaction.

EXPERIMENTAL

Sources of materials, preparation of "crude" and highly purified myoglobins, and experimental methods were described in a preceding paper (Brown and Dolev, 1963). In brief, "crude" beef and tuna myoglobin solutions were aqueous extracts of muscle that had been centrifuged and dialyzed against several changes of water. Purified myoglobins were prepared by ammonium sulfate fractionation of muscle extracts followed by DEAE-cellulose chromatography; these preparations were electrophoretically homogeneous. Formation of Mb^+ in solutions held at the indicated temperatures was determined spectrophotometrically by recording the spectral absorption curve in the visible region. Before being read, samples were thawed and/or brought to 10°C or higher in a water bath maintained at room temperature.

RESULTS AND DISCUSSION

All of the myoglobin solutions were oxidized more slowly at −5° than at 0°, al-

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though rates differed with buffer concentration. The rates were usually lower at higher buffer concentrations, particularly with beef myoglobin. At −5° none of the solutions were frozen. At −10°, however, the solutions were frozen and the rates of oxidation sharply increased. For most of the samples, the oxidation rate continued to increase as the temperature was lowered to −15°. For tuna myoglobin in 0.6*M* buffer, however, the rate was lower at −15° than at −10°; similarly, for two samples (beef and tuna myoglobins in 0.1*M* buffer) run at −18°, the rates of oxidation were lower than at −15°. Data are presented in Figs. 1 and 2. We interpret these results as indicating that as a result of the physical changes involved in freezing, rates of oxidation increase, in most cases very substantially. Note that the figures show logarithms of the rate constants. These increases in autoxidation rates presumably reflect the establishment of a more suitable myoglobin-oxygen environment. Under certain conditions there will be localized areas in which myoglobin will be concentrated during the freezing process; this is followed by localized thawing. On a number of occasions this phenomenon was observed in samples in this study; invariably, such samples oxidized at much higher rates. The increase in rate of oxidation brought about by the freezing process apparently begins to be counteracted by the negative effect of the lower temperature; hence, the observation in some of the samples of a "maximum" oxidation rate at some sub-freezing temperature.

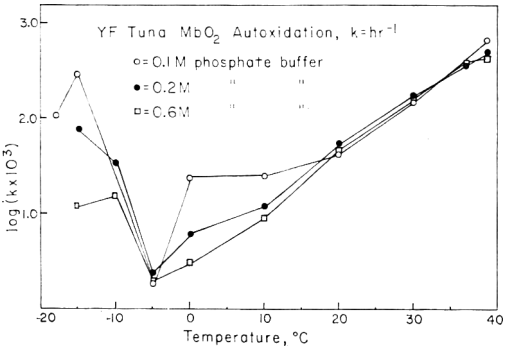

Fig. 1. Rates of oxidation of purified yellowfin tuna oxymyoglobin at various temperatures.

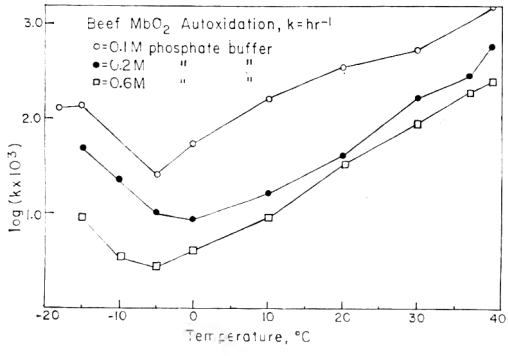

Fig. 2. Rates of oxidation of purified beef oxymyoglobin at various temperatures.

Samples of "crude" beef and tuna myoglobins similarly studied gave essentially the same results, except that the rates in every case at comparable buffer concentrations were always lower, reflecting the protective effect of proteins other than myoglobin in such extracts. These differences in rates between "crude" and purified myoglobins were greatest at higher temperatures, and at very low temperatures (−15°) were almost absent. "Crude" beef myoglobin in 0.6*M* buffer at −15° showed a reduction in rate of oxidation after several hours; both the initial rate and the secondary one were first-order, with the latter being slower. All other samples oxidized at a steady rate. In an effort to see if repeated freezing and thawing had an effect on autoxidation, replicate samples were taken from the same solution of "crude" beef myoglobin and held at −10°C. Some samples were repeatedly removed at various time intervals (for example at 1, 3, 5, 8 hr, etc.), whereas others were undisturbed for several hours before the first reading was made. The results are in Table 1. At −10° these solutions are just below their freezing point, and on certain occasions that could not be predicted or controlled, specific samples would be supercooled and not freeze. Readings were continued on these samples, and the results clearly indicate that at this low temperature the rate of autoxidation is extremely slow when the solution is not actually frozen, compared to a duplicate solution that has solidified. The results also indicate that repeated freezing and thawing does not have a strong effect on oxidation

Table 1. Autoxidation of replicate "crude" beef myoglobin samples at −10°C, 0.2*M* buffer, pH 5.9. Readings shown are absorbancies at 580 mμ; lowering indicates transformation of MbO_2 to Mb^+. Final value is after oxidation with ferricyanide.

	Time (hr)								
Sample	0	1	3	5	8	24	51	120	Final
A	.485	.425	.365	.330	.280	.270[a]	.265[a]	.255[a]	.110
B	.485	.....	.380	.335	.290	.180	.130	.....	.100
C	.485	.....	.....	.345	.295	.185	.155[a]	.115	.100
D	.485	.....	.....	.....	.305	.185	.130	.115	.100
E	.485	.....	.....	.....	.....	.190	.140	.115	.105
Av.	.485	.425	.372	.337	.292	.184	.139	.115	.103

[a] Sample did not freeze following the previous reading; these values not included in averages.

rate; of much greater importance is the fact that the solution has become solid.

Two constants can be calculated for solutions that did not freeze, since their autoxidation curves show sharp breaks. For the other samples in this series, calculated rate constants were 0.010–0.037 hr^{-1}. The significant increases in autoxidation rate noted as a result of freezing solutions of purified myoglobins suggests that this behavior is not without importance technologically.

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Electrophoretic Separation of Beet Pigments

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SUMMARY

Paper electrophoretic separation of pigments in beet extracts and in intact tissue was investigated. Pigments in an extract were separated into 9 distinct bands upon electrophoresis with 0.15*M* pyridine-citric acid buffer, pH 4.5. A technique is presented for the re-electrophoresis of pigments in paper section of an electrophoretogram. Using a veronal buffer (pH 8.6) during re-electrophoresis, heterogeneity of the pigments in major red and major yellow bands was displayed. Under specific conditions, beet pigments in a thin section of tissue could be resolved by electrophoresis.

INTRODUCTION

Simple methods for the separation of all pigments in the red root of the beet (*Beta vulgaris*) would be advantageous for determining the proportions of various pigments in the raw beet and for investigating the degradation of the coloring matter throughout the canning procedure. Beet pigments have been separated by the methods of column chromatography, column electrophoresis, and paper electrophoresis. According to Aronoff and Aronoff (1948), beet pigments in an extract were fractionated chromatographically on a talc-siliceous earth column into at least 11 colored bands. The pigments were isolated from the extruded column. By using the technique of column electrophoresis or modified column electrophoresis, the major red pigment called betanin has been recovered from other pigments (Lindstedt, 1956; Wyler and Dreiding, 1957; Peterson and Joslyn, 1960). Several investigators have reported that beet pigments can he separated on paper by electrophoresis. Schmidt and Schonleben (1956) found that betanin migrated toward the anode during electrophoresis when the pH of the buffer was above the isoelectric point (about pH 2). According to Lindstedt (1956), pigments of red beets were separated into at least 7 colored zones upon electrophoresis (6.3 V/cm) with a 0.1*M* citrate buffer, pH 5.5. Reznik (1957) showed that high-voltage electrophoresis (30–40 V/cm) was more effective than low-voltage electrophoresis (3.93 V/cm) for resolving beet pigments on paper. Nine colored bands, including a major violet-red zone and a major yellow zone, were formed when high-voltage electrophoresis was carried out with a phosphate buffer, pH 6.64, $\mu = 0.066$. With the aid of paper electrophoresis, Wyler *et al.* (1959) found that, along with betanin, 3 related violet compounds occurred in the red beet.

Low-voltage paper electrophoresis is a convenient, inexpensive method for fractionating electrically-charged constituents. Although several investigators have shown that both yellow and violet-red pigments of the beet can be separated into bands, detailed information on the resolution of red beet pigments by low-voltage paper electrophoresis with many types of buffers has not been reported. This investigation was undertaken to determine optimum conditions for the separation of pigments in beet extracts and in intact beet tissue by paper electrophoresis.

MATERIALS AND METHODS

Beet extract. Detroit Dark Red beets, with 3–3.5-in. diameters, were cut into small pieces and lyophilized. The lyophilized material was ground in a Wiley mill to pass a 60-mesh screen. The beet powder was stored in an air-tight container at −18°C.

Beet pigment extracts were prepared frequently with lyophilized beet powder throughout the experiment. To 1 g of lyophilized beet powder, 10 ml of 60% ethanol solution was added with stir-

ring. The mixture was allowed to stand 30 min at about 23°C, and was filtered through a sintered-glass filter under vacuum. The residue was extracted for 10 min with 10-, 5-, and 5-ml portions of 60% ethanol solution. To the combined filtrates was added 150 ml of anhydrous ethyl ether. After mixing in a separatory funnel, the mixture was allowed to stand for 1 hr at about 23°C. The concentrated pigment extract was placed in another funnel with 10 ml of 99% methanol. After the addition of 100 ml of anhydrous ethyl ether, the mixture was shaken vigorously and allowed to stand for 15 min at room temperature. The extract, removed from the ether, was concentrated to about 1.7 ml by passing dry nitrogen gas over the surface of the extract. The extract was stored under nitrogen at −18°C.

Beet tissue sections. Both fresh red beets of an unknown variety and frozen (−18°C for about 8 months) Ruby Queen beets were used for the preparation of beet tissue sections. Each beet sample, about 7 mm wide, 23 mm long, and 10 mm deep, was frozen to a CO_2 freezing chamber of a microtome. After partial thawing of the tissue, thin sections were cut with a razor blade mounted on a Spencer microtome.

Electrophoresis. The Spinco model R electrophoresis system was used exclusively in this study. Eight paper strips, S & S No. 2043 A, were used for each electrophoretic run. Electrophoresis was conducted either at room temperature (about 23°C) or at 3°C with a voltage gradient (V/l) of 5.2 volts/cm. Paper strips, saturated with buffer, were held for at least 20 min at 23°C or 12 hr at 3°C prior to sample application. To each strip, 0.02 ml of beet extract was applied by a sample applicator.

For re-electrophoresis of soluble pigments in a paper section of an electrophoretogram, the pigments were concentrated first in a small area by capillary flow of the selected buffer according to the following procedure. A piece of S & S no. 2043 A paper, 30 × 45 mm, was placed on a silicone-treated glass plate (small dimension of paper at top) resting on the inside wall of a 250-ml beaker containing the selected buffer. The buffer was allowed to flow up the paper while the strip was immersed to the depth of 5 mm. The immersed, buffer-saturated paper strip was held at 23°C for at least 5 min. Each pigment fraction was cut from the electrophoretogram so that 5 mm of unpigmented paper was included at one end of the section. This unpigmented portion of the section was pressed onto the top of the buffer-saturated filter paper. To hasten the upward movement for concentration of the pigment, dry nitrogen was trained on the upper end of the section. A glass plate was used to prevent gas flow on the lower portion of the section. The narrow pigmented segment (about 5 mm wide) was cut from the upper end of the section and pressed firmly onto a buffer-saturated strip in an off-center position between glass support rods. The adhesion of the pigmented segment to the paper strip was facilitated by applying 0.05 ml of buffer at the desired position just prior to placement of the segment. The electrical polarity was selected so that the pigments migrated down the paper strips.

Beet-pigment tissue sections were placed on buffer-saturated paper strips in an off-center position between two glass support rods in the ridge-pole electrophoretic cell. The tissue was pressed firmly to the paper with a metal spatula. The sections were allowed to equilibrate with the buffer for about 15 min prior to electrophoresis. Following each run, the strips were dried under a fan at room temperature (about 23°C). The dried strips were scanned immediately by an Analytrol densitometer, Model RB, having 550-mμ interference filters, 1.5 mm-slit width, and a B-2 cam. The Analytrol recorded the optical density between 0 and 1.5 for pigments along each strip.

The absorption spectra of beet pigments were determined with a Beckman DK-2 spectrophotometer. For this purpose, the beet pigments were eluted from paper sections with 0.1*M* citrate-phosphate buffer, pH 5.0.

RESULTS AND DISCUSSION

Electrophoretic separation of pigments in beet extract. For the preparation of a beet pigment extract, lyophilized beet powder was extracted with 60% ethanol so that extraneous matter such as proteins (including enzymes) and polysaccharides would be precipitated. No attempt was made to remove the large amount of sucrose in the beet extract. Sucrose, being a non-migrating constituent under the experimental conditions, was considered to be unobjectionable even though it was responsible for the high viscosity of the concentrated extract. Presumably the naturally-occurring pigments were extracted effectively with the 60% ethanol solution since they could not be detected in the cellulosic residue. As shown in Fig. 1, the visible absorption spectrum of beet pigments in a buffered extract (pH 5) had two major peaks, at about 478 and 530 mμ. Judging from the absorption spectra of isolated beet pigments (Aronoff and Aronoff, 1948; Wyler and Dreiding, 1957),

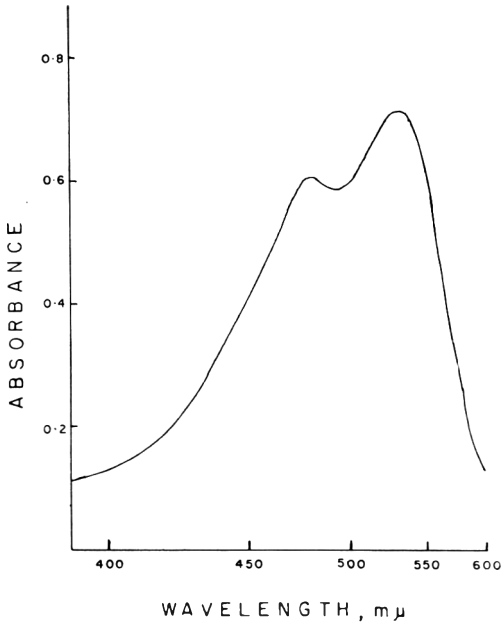

Fig. 1. Absorption spectrum of beet pigments in a buffered extract (pH 5.0).

the two maxima can be attributed to the high concentrations of both yellow-orange and red pigments.

During paper electrophoresis, all of the beet pigments migrated toward the positive electrode when the buffers used had pH values between 4.5 and 8.6. With buffers including phosphate (pH 6.5, 7.4), phosphate–citric acid (pH 4.6), pyridine–acetic acid (pH 4.8), and pyridine–citric acid (pH 4.5), two major pigment bands—red (R) and yellow (MY)—were separated, along with 3–7 minor yellow, pink, and orange bands. A non-mobile brown band was apparent at the origin of each electrophoretogram. This brown pigment undoubtedly was the result of oxidation of phenolic compounds. As shown in Fig. 2, the sequence of pigments from the origin was similar when the above-mentioned buffers were used for electrophoresis. However, with phosphate and phosphate–citric acid buffers, the electrophoretograms had an overlapping of the major yellow band with a minor pink band (Fig. 2-A,B,C). This overlapping was obviated by using pyridine as the base in a buffer. The pyridine–acetic acid buffer, pH 4.8, was satisfactory from the standpoint of preventing the pink-yellow overlap, but the major red pigment tailed excessively. Pigment resolution was much better when 0.15*M* pyridine–citric acid buffer was used instead of pyridine–acetic acid buffer. Resolution was improved further when electrophoresis was conducted at 3°C instead of room temperature. For instance, pigment boundaries were irregular when electrophoresis was conducted at about 25°C, because of excessive evaporation created by high buffer conductivity. In contrast, band boundaries were straight and pigments were resolved into 9 distinct zones with the buffer at 3°C (Fig. 2-E). The Analytrol trace (Fig. 3) of the above-mentioned electrophoretogram shows 4 peaks, which represent 4 red pigments. Sharp peaks are not apparent for the two orange pigments. This trace illustrates a typical relation of minor amounts of orange and red (pink-colored bands) pigments in contrast to the large quantity of pigment in the major red pigment band (R). Wyler *et al.* (1959) reported that, in addition to betanin, 3 re-

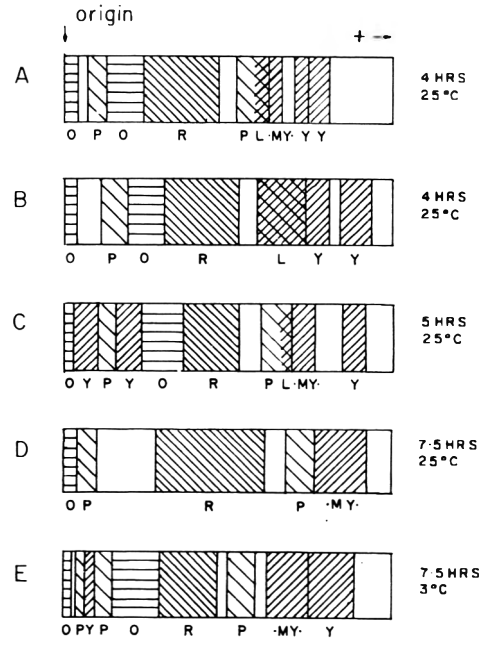

Fig. 2. Electrophoretograms of beet pigments. A) phosphate buffer, pH 6.5, $\mu = 0.1$; B) phosphate buffer, pH 7.4, $\mu = 0.1$; C) phosphate–citric acid buffer, pH 4.6, 0.1*M*; D) pyridine–acetic acid buffer, pH 4.6, 0.1*M*; E) pyridine-citric acid buffer, pH 4.5, 0.15*M*. Band color: O–orange, P–pink, R–red (major red), Y–light yellow, MY–deep yellow (major yellow), L–overlap of pink (P) and deep yellow (MY).

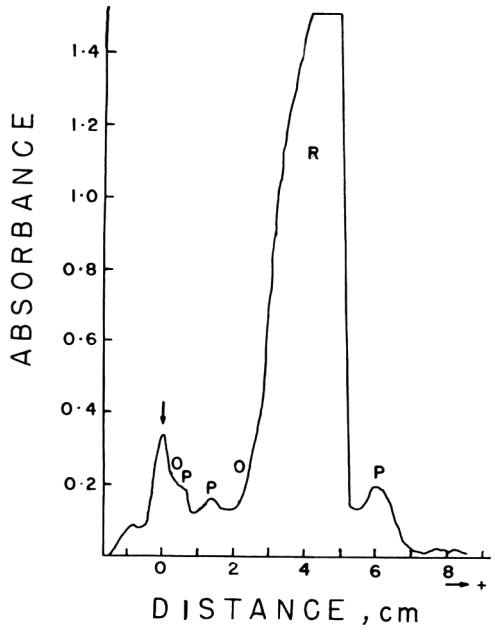

Fig. 3. Analytrol trace of beet pigment electrophoretogram with pyridine–citric acid buffer, pH 4.5, 0.15*M* (electrophoresis for 7.5 hr at 3°C). Band color: O–orange, P–pink, R–red.

lated violet pigments were present in the red beet. Yellow pigments do not absorb significant amounts of light energy in the 550-mμ region, and consequently cannot be detected in the trace. The high optical-density value at the origin of the electrophoretogram is due to the presence of brown pigments.

With a veronal buffer (pH 8.6, $\mu = 0.075$) one major purplish-red and two major yellow bands, along with minor purplish-red and yellow bands, were obtained during electrophoresis. No orange bands were detected under these buffer conditions. An advantage for the use of veronal buffer rather than buffers with lower pH values is that the major red pigment (R) migrated in a narrow band, with almost all of the yellow pigments in preceding bands.

Under specific electrophoretic conditions, the electrophoretograms could be reproduced. The standard deviations of the mean values for migration distance (M) and band width (BW) of pigments on electrophoretograms were very small.

Mobility and band width of the beet pigments are influenced by the type and ionic strength of the buffer. Table 1 indicates that the migration of pigments increased with a small pH increment ($\triangle 0.9$) of the phosphate buffer, $\mu = 0.1$. Except with the major red band (R), the zones were broader at the higher pH value. With an increase in pH, the negative charge on the pigment molecules undoubtedly increases. According to Mabry *et al.* (1962), three ionizable carboxyl groups are present in betanin. Thus, a decrease of hydrogen ions in a buffer would provide greater negativity to the betanin because of greater dissociation of the carboxyl groups.

Table 2 indicates that the ionic strength of the phosphate-citrate buffer influences the migration distance (M) and band width (BW) of some beet pigments. The migration distance and band width of the minor pink band near the origin were not influenced markedly by an alteration of ionic strength. When ionic strength was increased to 0.167, excessive evaporation caused an irregular major red band. The narrow major red bands (R) on electrophoretograms obtained with buffers having ionic strengths of 0.1 and 0.167 permitted detection of an orange-yellow zone, just behind the major red band (R). The band width of the major yellow pigment could not be determined, because the overlapping pink band obscured the tailing boundary of the yellow pigment.

In an effort to determine the heterogeneity of the major red (R) and major yellow (MY) bands of the electrophoretogram obtained with 0.15*M* pyridine–citric acid buffer (pH 4.5), re-electrophoresis of these pigments was carried out with veronal buf-

Table 1. The influence of buffer pH on the electrophoretic migration (M) and band width (BW) of beet pigments (in mm).

Band color	Buffer [a]			
	A (pH 6.5)		B (pH 7.4)	
	M [b]	BW [b]	M	BW
Pink	16	7	21	8
Major red	55	25	59	26
Pink	73	12	83	19
Major yellow	77	.....	83	.....
Yellow	81	4	91	8
Yelow	94	7	106	10

[a] Phosphate buffer, $\mu = 0.1$.
[b] Mean for 6 strips.

Table 2. Influence of ionic strength on the electrophoretic migration (M) and band width (BW) of beet pigments (in mm).

Band color	Ionic strength [a]							
	0.025		0.05		0.1		0.167	
	M [b]	BW [b]	M	BW	M	BW	M	BW
Pink	19	8	20	9	18	5	17	8
Major red	74	45	73	29	59	17	44	14
Pink	97	16	95	13	77	8	60	10
Major yellow	102	.....	102	.....	83	.....	67	.....

[a] Phosphate–citrate buffer, pH 4.6.
[b] Mean for 6 strips.

fer, pH 8.6. Prior to re-electrophoresis the major red band (R) was divided into a highly pigmented zone (R_1) and a less pigmented tailing zone (R_2). With zone R_1, one purplish-red band and two minor yellow bands were separated electrophoretically. The tailing zone (R_2) yielded a purplish-red band and four light yellow bands. Pigments in the major yellow band (MY) were separated electrophoretically into one deeply-colored yellow band and one light-yellow band. Table 3 shows the migration distances (M) and band widths (BW) for the pigments in the major red (R) and yellow (Y) bands.

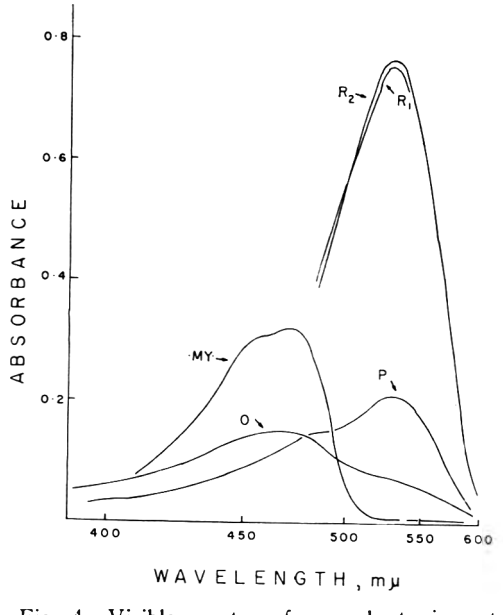

Fig. 4. Visible spectra of some beet pigments eluted (citrate buffer, pH 5.0) from bands of electrophoretograms (0.15*M* pyridine–citric acid buffer, pH 4.5). Band color: O–orange, P–pink, (near MY band), R_1–red, R_2–red, MY–deep yellow.

Fig. 4 shows the visible spectra of some pigments eluted with citrate buffer (pH 5) from sections of electrophoretograms obtained with 0.15*M* pyridine–citric acid buffer (pH 4.5). The pigments in zones R_1 and R_2 of the major red band (R) had similar visible spectra with maximum absorption at about 530 mμ. With pure betanin, the (visible) absorption spectra as reported by Wyler and Dreiding (1957) and Peterson and Joslyn (1958) were similar to that in Fig. 4. The assumption may be made that the red pigment in the major red band (R) is betanin. With eluted pigments from the major yellow band, a spectrum was obtained having a peak between 460 and 470 mμ. In the ultraviolet region, all of the eluted red, yellow, and orange pigments had the same spectra, with 2 peaks at about 250 and 255 mμ. A small amount of pyridine,

Table 3. Results (in mm) of re-electrophoresis of the major red and major yellow bands of the electrophoretogram obtained with 0.15*M* pyridine–citric acid buffer (pH 4.5).

Electrophoretic sections [a]	Band color	M [b]	BW [b]
Major red, R_1	purplish-red	59	9
	yellow	65	6
	yellow	89	7
Major red, R_2	yellow	20	9
	yellow	34	10
	purplish-red	56	16
	yellow	62	6
	yellow	71	3
Major yellow	yellow	62	6
	yellow	68	3

[a] Obtained from electrophoretograms, using veronal buffer, pH 8.6, $\mu = 0.075$.
[b] Mean for 3 strips.

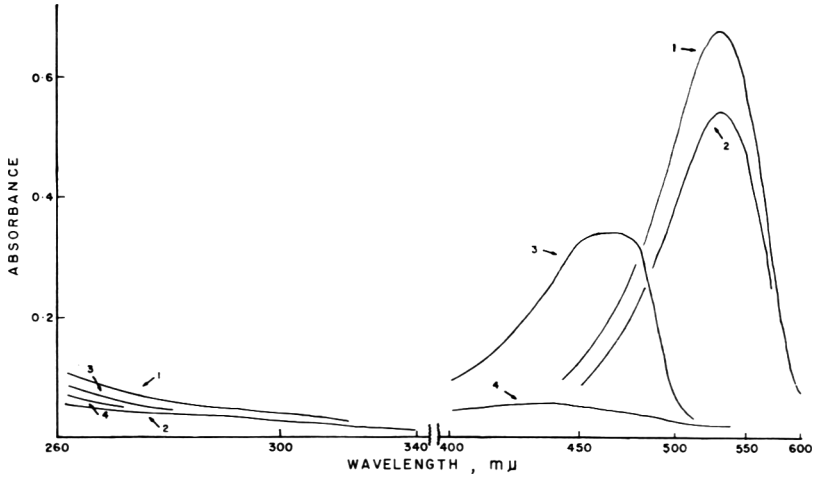

Fig. 5. Spectra of some pigments eluted from re-electrophoretograms (veronal buffer, pH 8.6, $\mu = 0.075$). 1) red pigment from R_1 band; 2) red pigment from R_2 band; 3) deep yellow pigment from MY band; 4) light yellow pigment from MY band.

eluted from the paper sections, proved to be responsible for these peaks.

The spectra of some pigments eluted from re-electrophoretograms are presented in Fig. 5. As might be expected, the visible spectra of the purplish-red pigments from zones R_1 and R_2 had maximum absorption at about 530 mμ. In the region between 260 and 340 mμ, no appreciable absorption was exhibited with these red pigments. However, in the ultraviolet spectrum of crystalline betanin prepared by Wyler and Dreiding (1957) and Peterson and Joslyn (1960), a slight amount of absorption was shown in the region between 240 and 300 mμ. Peterson and Joslyn (1958, 1960) implied that an absorption band in the ultraviolet spectrum of betanin is an impurity. A crude fraction of the major red beet pigment displayed marked ultraviolet absorption in the region of 240–300 mμ (Aronoff and Aronoff, 1948). The deep-yellow band of the re-electrophoretogram for the major yellow band (MY) had an optimum absorption at about 465 mμ. The absorption band for the minor yellow pigment from the major yellow band (MY) was very broad and had an optimum absorption between 435 to 445 mμ.

Electrophoretic separation of pigments in beet tissue. The electrophoretic separation of pigments from intact beet tissue involves very few simple techniques, including the cutting of tissue into thin sections with a microtome and applying the sections on buffer-saturated paper. The thickness and width of the sections are critical for successful resolution of the pigments and prevention of tailing. A section suitable for electrophoresis must be flexible enough to adhere completely to the buffer-saturated paper and must contain a sufficient amount of minor pigments so that the minor bands can be detected. The optimum thickness of a tissue section with a width of about 7 mm was found to be about 200–240 μ. Tailing of pigments was excessive when the thickness of the tissue was much greater than about 240 μ.

Using a 0.15M pyridine–citric acid buffer, pigments in sections cut from fresh beets of an unknown variety were separated electrophoretically into 7 distinct bands (Fig. 6). The tissue sections after electrophoresis

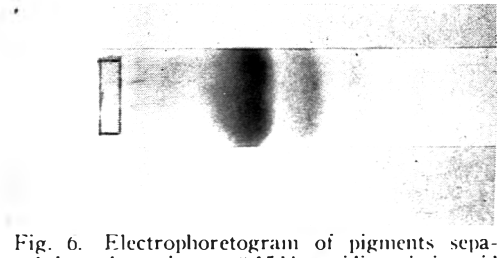

Fig. 6. Electrophoretogram of pigments separated from beet tissue (0.15M pyridine–citric acid buffer, pH 4.5).

were slightly brown, but no naturally-occurring pigments could be detected visually. When fresh tissue was frozen 44 hr in a freezer at −18°C, the pigments migrated on paper, during electrophoresis, more rapidly than pigments from unfrozen tissue. The disruption of the cellular structure by ice crystals was presumably responsible for the improvement in migration.

Even with tissue from Ruby Queen beets that had been frozen 8 months at −18°C, resolution of pigments was excellent during paper electrophoresis. The Analytrol trace of an electrophoretogram with Ruby Queen beet pigments is presented in Fig. 7. Definite peaks representing the red and orange pigments are shown in the trace. Major red and major yellow bands were cut from each of 3 beet-tissue electrophoretograms. The pigments were eluted with 0.1*M* citrate buffer, pH 5.0, and solutions were brought up to a specific volume (4 ml for the major yellow pigment and 10 ml for the major red pigment). Optical density values were determined at 530 mμ for red-pigment solution and at 470 mμ for the yellow solution. The values of ratio OD_{530}/OD_{470} for the 3 electrophoretograms were 3.2, 3.2, and 3.5. Valuable information on the degradation of major pigments by physical and chemical treatments could undoubtedly be obtained from the pigment optical-density ratios.

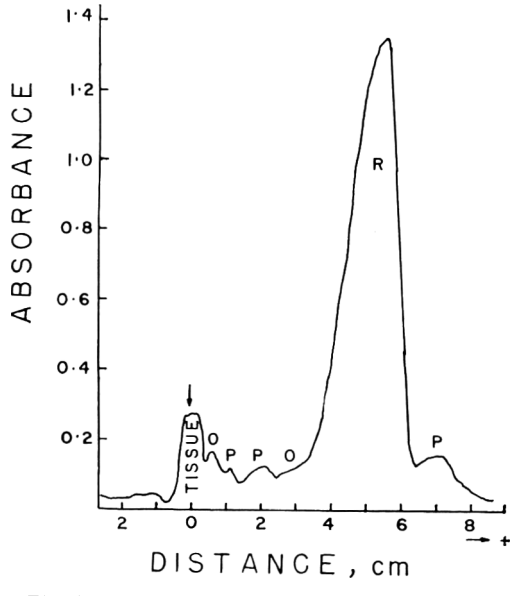

Fig. 7. Analytrol trace of a beet-tissue electrophoretogram with 0.15*M* pyridine–citric acid buffer, pH 4.5 (Ruby Queen beets frozen for 8 months at −18°C). Band color: O–orange, P–pink, R–red.

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Chemical and Radiochemical Composition of the Rongelapese Diet

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SUMMARY

The gross chemical composition of the Rongelapese diet indicates that it is low in fat, protein, and ash but fairly high in carbohydrate. The variation in gross chemical composition of the diets examined may be accounted for by the broad variability of the different diets. The habitat of the Rongelapese probably does not demand a high-energy diet, which may partially justify the lower fat intake. Levels of calcium and phosphorus seem below the minimum required for maintenance of a proper calcium-phosphorus balance. The diet seems adequate in magnesium and potassium but slightly low in sodium. The nickel, cobalt, and copper contents seem high in the Rongelap rations, manganese content is low, and iron and zinc compare favorably with minimum daily requirements.

Hight levels of cobalt-60 and zinc-65 are associated with each other and with rations containing local fish. The higher levels of strontium-90 and cesium-137 are found where local fruit was consumed. Coconut contributes little strontium-90, and pandanus the most. Rations with higher zinc-65 also contain higher levels of stable zinc, indicating that local sea foods may he the main source of zinc in the diet. Cesium-137, strontium-90, and cobalt-60 show no definite correlation with stable potassium, calcium, and cobalt, respectively. There is probably a net addition of minerals to Rongelap soils from imported foods.

INTRODUCTION

Rongelap Atoll was contaminated with radioactive fallout resulting from the Bravo test, on March 1, 1954, to the extent that the population of 82 Rongelapese had to he evacuated. Some 200 Marshallese returned to Rongelap in June, 1957, after the area had been declared again safe for human habitation. Since 1954 several surveys have determined levels of radioactive contamination at Rongelap Atoll (Dunning, 1957). In March, 1958, a study of the ecology of the atoll relative to radioactive contamination was initiated at the request of the U. S. Atomic Energy Commission, Division of Biology and Medicine.

One of the objectives of the present investigation was to determine the amount and kinds of radionuclides and minerals ingested by the Rongelapese through foods. Fat, protein, and carbohydrate were determined to provide a basis of comparison with known diets. To our knowledge there are no published data on the diet of the Rongelapese.

Rongelap Atoll lies in the northern Marshall Islands, an area of comparatively low rainfall and limited variety of agricultural products. The principal plants eaten are coconut, breadfruit, pandanus, and the arrowroot, or tacca; some squash and papaya are also grown. Bananas and taro have been introduced but are not yet in full production.

Fish, clams, langusta, birds, chickens, and pigs are eaten. Of these, the most important is fish. The coconut crab, *Birgus latro*, is considered a delicacy but is the one food item excluded from the diet because of the strontium-90 content (Dunning, 1957).

The coconut, "Ni" in Marshallese, is eaten at different stages of development. The juice

[a] Operated by the University of Washington under Contract No. AT (45-1) 1385 with the United States Atomic Energy Commission.

from the immature nut is preferred for drinking, and only rarely is the germinated nut eaten. Copra, the dried coconut meat, is a staple eaten alone or mixed with other foods.

The coconut sap is collected from the cut ends of inflorescences of coconut trees set aside for this purpose in the village area. The fresh sap is partaken of by all age groups, and the fermented sap, "jekro" or "jugaroo," is consumed by adults.

Breadfruit (Ma) is eaten either baked or boiled, and also is made into a preparation referred to as "cheese." To make "cheese," the skin is removed and the pulp is placed in salt water for three days, then wrapped in breadfruit leaves and buried in the sand for at least one week before it is eaten. The Rongelapese claim that this "cheese" will keep two years or more in the sand.

Pandanus (Bop) is eaten fresh, boiled, or baked. "Jenkun," a preparation said to keep for more than five years, is prepared by baking or boiling the Pandanus keys and scraping out the pulp. The pulp is dried, usually on sheet metal, over coals until it reaches the consistency of fudge. The dried pulp is pressed into a roll and wrapped in Pandanus or coconut leaves. Slices are cut off as needed.

Tacca or arrowroot (Mokmok) tubers are washed with sea water, crushed and passed through a sieve into a pan of sea water, and allowed to settle for three or four hours. When the starch begins to coagulate, the water is decanted. Sea-water washes are repeated several times, followed by one fresh-water wash. Finally the starch is dried and stored as a powder. The powder is mixed with water for use, and either eaten immediately as prepared or boiled or baked.

The papaya is eaten fresh, sometimes mixed with rice or grated coconut.

The fish consumed are primarily reef fish such as the goatfish, *Mulloidichthys* sp., mullet, *Neomyxus* sp., surgeon-fish, *Acanthurus* sp., and the siganids. The fish are eaten baked, boiled, or raw. The three fish we have observed the Rongelapese to eat raw are goatfish, mullet, and siganids. Fish are also preserved by baking and then drying in the sun. Sometimes salt is added before the fish are dried.

The langusta, spiny lobster, is eaten boiled. The clams are either boiled as a chowder or baked in the shell in a covered pit. The clam meat is sometimes also dried in the sun after baking and kept for several days.

Birds are eaten either baked or boiled, and are also dried following precooking. Bird eggs, usually hard boiled, form only an incidental part of the diet; they are used principally when the Rongelapese are visiting islands other than their main island or nearby Ailinginae Atoll.

Pig and chicken are eaten primarily on special occasions.

The source of fresh water in the area is cisterns. Ground water, though potable in certain areas during the rainy season, is not ordinarily drunk.

Of the imported foods, rice, wheat flour, and canned corned beef appear to be the most important. Many other products are imported from time to time, such as sardines, C-ration, ship's biscuit, and candy. In 1958 large quantities of C-rations were consumed. Many individuals prefer the imported foods.

MATERIALS AND METHODS

The samples were collected during a single 24-hr period in September, 1959, at Rongelap Island, taking care that the composition and the amount corresponded to the composition and amount actually eaten by the individual. (Bwio Soap, former village secretary, and Neil Morriss, Trust Territory Resident Agriculturist at Rongelap Atoll at that time, or one of the authors collected each sample.) Wet weights of the samples were taken in the field. The samples were then dried at 90°C and shipped to the University of Washington, Seattle.

Caution must be used in collecting to be reasonably sure that such daily rations are a true representation. Misunderstanding and a misguided desire to please can easily lead some Rongelapese to provide merely a collection of miscellaneous food items rather than actual daily rations of prepared food. It was felt that a few samples composed of items and portions actually seen to be consumed were preferable to many samples of uncertain origin. Consequently, some samples proffered by individuals were discarded.

Even so, there are obvious discrepancies. Sample number 3 (Table 1), for example, appears to be ridiculously low in the total amount consumed. Doubtless there must have been some "snacking," but the eating habits of the Rongelapese are irregu-

Table 1. Description of food rations collected at Rongelap Island in September, 1959 (each sample is a 24-hr ration).

Sample no.	Description	Wet wt. (g)	Total dry wt. (g)
1	a) Pandanus paste, boiled rice and baked fish (mixed)	253	374.68
	b) Partly baked bread dough with bully beef	252	
	c) Bully beef sandwiches	195	
2	a) Coconut meat (green)	30	175.85
	b) Pandanus "pie"	16	
	c) Baked fish	23	
	d) Sardines, canned	20	
	e) Boiled rice w/coconut milk	249	
3	a) Breadfruit, baked	41	87.12
	b) Coconut and bread dough, baked	24	
	c) Bread	31	
	d) Bully beef	17	
	e) Ship's biscuit	13	
	f) Rice w/coconut milk, boiled	49	
4	a) Coconut, ripe	72	321.69
	b) ½ papaya	57	
	c) Rice and fish mixed	306	
	d) Bread, local (coconut milk, not saved)	81	
5	a) Rice and fish mixed	243	203.16
	b) Bread, local	80	
	c) Rice	197	
6	a) Breadfruit, baked	203	484.10
	b) Coconut w/baked dough	203	
	c) Fish, baked	126	
	d) Bread, local	75	
	e) Coconut, entire	50	
	f) Rice, boiled	291	
	g) Sardines, canned	154	
7	a) Pandanus keys, raw	115	314.90
	b) Goatfish, baked	26	
	c) Sardines, canned	101	
	d) Rice, boiled	721	
8	a) Fish, baked	155	440.50
	b) Bread, local	145	
	c) Bully beef	66	
	d) Sardines, canned	94	
	e) Rice, boiled	622	
9	a) Rice and fish mixed	421	262.30
	b) Rice and fish mixed	64	

lar and it was impractical to follow each individual throughout the day. Therefore, all of the samples collected (Table 1) should probably be considered as erring toward the low side for total consumption. However, there does appear to be a reasonable agreement with quantities listed by Murai (1954) from a study at Majuro Atoll. Catala (1957) pointed out the difficulties of obtaining quantitative data in these areas.

The components of each sample were dried to constant weight in the laboratory at Seattle (Table 1). The entire diet for each individual was then homogenized in water with a high-speed blender, dried at 98°C, and pulverized to a fine powder. Subsamples of the powder were taken for fat, protein, carbohydrate, and radiochemical analyses. Portions weighing 40–250 g were wet-ashed with HNO_3 and H_2O_2, and the ash dried in 250-ml beakers for gamma-ray spectroscopy.

The gamma-counting equipment consisted of a 3-in. thallium-activated sodium iodide crystal used in conjunction with a 256-channel analyzer with a digital print-out. The total counts per minute under the photopeak were calculated by summing counts per minute of all channels included in the peak and subtracting the background counts. The counting efficiency for the gamma energies measured was determined by calibrating the instrument with standards with an error of $\pm 10\%$.

Following analysis by gamma spectroscopy, the ashed samples were dissolved in a known volume of 1*N* HNO_3. Strontium-90 was determined on an aliquot by the method of Kawabata and Held (1958), in which a combination of nitric acid precipitation and ion-exchange procedures is used.

Calcium was determined by permanganate titration of oxalic acid and confirmed by flame spectrophotometry, with the internal standard technique of Chow and Thompson (1955). Potassium was determined by flame spectrophotometry at 766-mμ wavelength, and independently confirmed with estimation of potassium by titration of the cobaltinitrite with potassium permanganate (Hibbard and Stout, 1933). Sodium was determined at 589-mμ wavelength. The standardization procedure and general function of the system have been described by Chakravarti and Joyner (1960).

In determining magnesium, an aliquot of the ashed sample was dissolved in 0.1*N* HCl and the solution passed through a Dowex-50 X8 100-200 resin column of precalculated capacity. Interfering anions were removed by elution with two-column volumes of distilled water. The resin was then stripped of cations with three-column volumes of 2*N* HCl, and the eluate was neutralized to methyl orange with concentrated NH_4OH. Calcium was removed by precipitation with ammonium oxalate followed by boiling and filtration. The filtrate was

Table 2. Composition of rations from Rongelap Island (dry-weight basis).

Constituent		Sample no. 1	2	3	4	5	6	7	8	9
Proximative analyses (%)										
Moisture		46.5	48.0	50.2	37.7	60.9	59.7	67.3	59.3	45.9
Fat		3.73	1.64	1.35	1.34	4.35	8.47	2.80	3.60	2.82
Protein		15.1	10.7	11.6	9.65	7.65	23.7	14.5	25.9	8.65
Carbohydrate		30.5	37.8	32.7	49.2	21.3	3.57	10.9	6.98	38.9
Ash		4.17	1.87	4.13	2.19	5.83	4.62	4.54	4.28	3.71
Chemical composition										
Calcium	mg/g	0.761	0.593	0.920	0.571	0.381	2.13	1.29	0.624	0.455
Magnesium	mg/g	0.804	0.797	1.13	0.938	0.777	1.10	0.657	0.760	0.814
Sodium	mg/g	3.42	2.44	6.20	1.97	2.59	4.57	7.32	3.22	2.77
Potassium	mg/g	2.28	1.39	3.12	3.34	1.12	4.60	2.55	2.95	1.52
Phosphorus	mg/g	0.134	0.061	0.024	0.119	0.056	0.358	0.203	0.823	0.102
Nitrogen	mg/g	24.2	17.1	18.5	15.5	12.3	37.8	23.9	41.3	13.8
Nickel ppm	(mg/kg)	0.0	24.	4.6	1.7	33.	5.4	25.	3.2	1.7
Manganese	(mg/kg)	.71	1.0	.22	2.7	2.5	2.9	3.3	2.2	1.7
Cobalt	(mg/kg)	2.1	.80	.30	.63	.00	.27	.33	.12	.29
Copper	(mg/kg)	14.	27.	8.9	20.	5.6	22.	7.5	6.8	2.8
Iron	(mg/kg)	66.	69.	44.	34.	33.	47.	33.	71.	29.
Zinc	(mg/kg)	24.	14.	16.	13.	16.	48.	37.	41.	29.

Table 3. Composition of rations for a 24-hr period from Rongelap Island.[a]

Constituent		Sample no. 1	2	3	4	5	6	7	8	9
Wet wts.	(g)	700.0	338.0	175.0	516.0	520.0	1201.0	963.0	1082.0	485.0
Moisture	(g)	325.	162.	87.9	194.	317.	717.	648.	641.	223.
Fat	(g)	14.0	2.88	1.17	43.2	8.84	41.0	8.82	15.8	7.38
Protein	(g)	56.6	18.8	10.1	31.0	15.5	114.	45.5	114.	22.7
Ash	(g)	15.6	3.29	3.59	7.06	11.8	22.4	14.3	18.8	9.74
Carbohydrate	(g)	288.	151.	72.3	240.	167.	306.	246.	292.	222.
Calcium	(g)	0.285	0.104	0.080	0.184	0.077	1.03	0.407	0.275	0.119
Magnesium	(g)	0.301	0.141	0.088	0.302	0.158	0.531	0.207	0.335	0.214
Sodium	(g)	1.28	0.429	0.540	0.634	0.526	2.21	2.30	1.42	0.727
Potassium	(g)	0.854	0.244	0.272	1.07	0.228	2.23	0.803	1.30	0.399
Phosphorus	(g)	0.036	0.012	0.002	0.038	0.011	0.173	0.064	0.080	0.027
Nitrogen	(g)	9.06	3.00	1.61	4.99	2.49	18.3	7.52	18.2	3.62
Nickel	(mg)	0.0	.91	.40	.51	6.7	2.6	7.7	1.4	.45
Manganese	(mg)	.27	.18	.02	.86	.50	1.4	1.0	.99	.45
Cobalt	(mg)	.78	.14	.03	.20	0.0	.13	.10	.05	.07
Copper	(mg)	5.3	4.7	.77	6.3	1.1	11.	2.4	3.0	.73
Iron	(mg)	25.	12.	3.9	11.	6.7	23.	10.	31.	7.5
Zinc	(mg)	8.9	2.4	1.4	4.3	3.2	23.	12.	18.	7.7

[a] Calculated from Table 2, wet-to-dry ratio, and weight of total sample.

Table 4. Radioisotopes (disintegrations per minute per gram) in rations from Rongelap Island (dry-weight basis).

Sample no.	Co^{60}	Zn^{65}	Mn^{54}	Cs^{137}	Sr^{90}
1	0.35 ± .12[a]	0.40 ± 0.29	−0.04 ± 0.09	61.4 ± 0.60	0.84 ± 0.07
2	0.52 ± .25	−1.03 ± 0.53[b]	0.11 ± 0.20	14.1 ± 0.50	1.63 ± 0.16
3	0.12 ± .52	−2.40 ± 1.0	−0.49 ± 0.39	21.1 ± 0.87	1.25 ± 0.25
4	0.23 ± .13	−0.76 ± 0.28	−0.07 ± 0.10	17.6 ± 0.38	0.43 ± 0.06
5	0.43 ± .22	−0.67 ± 0.45	−0.11 ± 0.16	3.6 ± 0.28	0.21 ± 0.09
6	0.90 ± .13	1.70 ± 0.30	−0.23 ±0.09	16.1 ± 0.29	0.66 ± 0.06
7	0.56 ± .14	0.87 ± 0.35	−0.19 ± 0.10	20.0 ± 0.38	0.86 ± 0.08
8	1.20 ± .15	2.50 ± 0.41	−0.21 ± 0.11	3.0 ± 0.17	0.22 ± 0.05
9	0.33 ± .16	0.05 ± 0.36	−0.003 ± 0.13	2.6 ± 0.21	0.32 ± 0.08

[a] 0.95 counting error.
[b] Negative values are given to indicate that there are errors in addition to the counting error which cannot be specifically accounted for.

made basic with 1*N* NH_4OH; 5% $(NH_4)_2PO_4$ was added until a precipitate formed, and an excess of NH_4OH was then added during constant stirring. The precipitate, magnesium ammonium phosphate, was allowed to settle overnight, removed by filtering, dissolved in 6 drops of concentrated H_2SO_4, and made to volume with water. Magnesium was determined by titrating an aliquot of this solution against a standard EDTA solution using the indicator Eriochrome Black T.

Total phosphorus was determined by the colorimetric method of Fleischer *et al.* (1958).

The transition elements nickel, manganese, cobalt, copper, iron, and zinc were determined colorimetrically by methods described by Sandell (1959). The elements were initially separated by selective elution of their chloride complexes from an anion-exchange resin. Kraus and Moore (1953) have shown that the chloride complexes of the transitional elements nickel through zinc are adsorbed onto a strongly basic anion-exchange resin (Dowex 1) and are selectively eluted at different molarities of HCl. Following the same principle, Joyner and Chakravarti (1960) suggested techniques that were applied to these samples.

Protein nitrogen was determined by the Kjeldahl method.

Fat was determined by a modification of the Johnson method (Winton and Winton, 1945). Methylene chloride was the extracting solvent.

Ash content was determined as the nitrate form by drying an aliquot of the ashed sample to constant weight.

Moisture content was calculated from the wet-weight to dry-weight ratio.

Total carbohydrate and like substances were estimated by subtracting moisture, fat, protein, and ash from the total solids and calculating the carbohydrate content by difference.

RESULTS AND DISCUSSION

Table 1 lists the components of the 24-hr food rations collected at Rongelap Island. Tables 2 and 3 show proximate composition and trace-element content of the rations, and Tables 4 and 5 present levels of radioisotopes. Results are given on a percentage or unit weight basis (Tables 2 and 4) and as amount for total diet (Tables 3 and 5). The former basis permits comparison of the relative composition of individual rations and facilitates evaluation of the contributions made by specific items in each diet; the latter basis shows the actual amounts consumed in a 24-hr period.

In evaluating the chemical constituents consumed by an individual in a 24-hr period, the gross weight of the total diet is of much importance. By comparing the proximate chemical composition on a percentage basis with the published chemical composition of some of the items constituting the samples, it is possible to account for the variation in moisture, fat, protein, carbohydrate and ash content of the different diets.

Since information on the nutritional aspects of the Rongelapese diet is limited, comparison of the data with data for other areas is probably not meaningful. The gross percentage composition indicates that the diets are generally low in fat, protein, and ash but fairly high in carbohydrate content.

When the data in Table 3 are compared with the recommended daily dietary allowances published by the Food and Nutrition Board of the National Research Council, the

24-hr rations of the Rongelapese appear to be generally below the level recommended for protein. Since fat allowances are based more on food habits than on physiological requirements, no definite conclusion can be drawn about the apparent low fat content of these diets. The habitat of the Rongelapese probably does not demand a high-energy diet, which may partially justify the lower fat intake.

The calcium content of the 24-hr ration seems to be much lower than the suggested normal requirement (Nutritional Data, 1958). On the same basis, the magnesium levels seem to be adequate but the phosphorus levels are far below what is necessary to maintain a proper calcium-phosphorus balance in a good diet. The sodium levels appear to be slightly below the normal suggested intake levels, although no information is available as to the minimum daily requirement of sodium. The potassium level is lower than the sodium content, which is generally the case in most diets.

Kent and McCance (1941) have suggested that an ordinary adult diet will supply 0.3–0.5 mg of nickel daily. On the basis of these values, the nickel content of the 24-hr Rongelapese rations appears to be higher than usual in some cases. Nickel salts frequently gain access to food from corrosion of nickel vessels, and small quantities of nickel may also be found in various manufactured foods. It also may be that some of the native food components are high in nickel content.

Basu and Malakar (1940) have suggested that 4.6 mg of manganese are required per day to keep an adult male in manganese balance. On this basis, the Rongelapese food appears to be low in manganese. The average adult diet of good quality supplies 0.005–0.008 mg of cobalt daily (Harp and Scoular, 1952); in comparison the Rongelapese food appears to be fairly high in cobalt content. Tomsett's (1934) balance experiments with adult humans indicate a minimum copper requirement as low as 0.6 mg daily. The estimate of Chou and Adolph (1935) is 1–2 mg daily. The Rongelapese diet is definitely above the experimental minimum requirements given. The iron in the diet appears to compare favorably with the minimum daily requirement as suggested by the National Research Council. Eggleton (1939) has given normal daily food intake of zinc as 12 mg. The Rongelapese food appears to have large variation in zinc content, and on the average is less than 8 mg daily.

The higher levels of cobalt-60 and zinc-45 are associated with each other and with rations containing local fish. This is to be expected since these isotopes are found primarily in marine organisms (Dunning, 1957). The higher levels of strontium-90 and cesium-137 are found where local fruit was consumed. In general, higher levels of strontium-90 are coincident with higher levels of cesium-137. Coconut contributes little strontium-90, and pandanus the most.

The average value for the daily intake of strontium-90 is 83 $\mu\mu$c, and for calcium 0.28 g. The average daily intake in terms of "strontium units" ($\mu\mu$c Sr^{90}/g Ca) is then nearly 300. This value is about three

Table 5. Radioisotopes in 24-hr rations from Rongelap Island.[a]

Sample no.	$\mu\mu$c/24 hr				
	Co^{60}	Zn^{65}	Cs^{137}	Sr^{90}	$\mu\mu$c Sr^{90}/g Ca
1	59 ± 20[b]	67 ± 49	10000 ± 100	142 ± 11.8	497 ± 41.4
2	42 ± 20		1100 ± 40	129 ± 12.7	1239 ± 121.6
3			830 ± 34	49 ± 9.8	613 ± 122.5
4	33 ± 19		2600 ± 55	62 ± 8.7	339 ± 47.3
5	39 ± 20		330 ± 26	19.2 ± 8.2	248 ± 106.5
6	200 ± 48	370 ± 65	3500 ± 63	144 ± 13.1	140 ± 12.7
7	79 ± 20	120 ± 50	2800 ± 54	122 ± 11.4	300 ± 27.9
8	240 ± 30	500 ± 82	590 ± 34	43.6 ± 9.9	159 ± 36.1
9	39 ± 19		310 ± 25	37.8 ± 9.5	331 ± 82.8

[a] Calculated from Table 4, wet-to-dry ratio, and weight of total sample.
[b] 0.95 counting error.

times that of previous estimates (Dunning, 1957; Cohn *et al.*, 1960). Those estimates were based on an estimated daily intake of 0.8 g of calcium, or about three times the value reported here. Thus, the significant difference between this and previous values reflects a discrepancy between observed and estimated calcium intake. It is not within the scope of this report to enter into an estimation of body burden, which has been discussed in detail by Cohn *et al.* (1960). However, it is of interest to note that the body burden as estimated from urinalysis data (Woodward *et al.*, 1959) and discussed by Cohn is consistent with a discrimination factor of four and a daily intake of about 100 $\mu\mu$c Sr^{90}/g calcium. This would indicate either that the discrimination factor is greater than four or that these samples do not correctly represent daily calcium intake. In any case, it is obvious that continued study of Sr^{90} movement at Rongelap Atoll is necessary.

Rations containing the higher levels of zinc-65 also contain the higher levels of stable zinc, indicating that local sea foods may be the main source of zinc in the diet. Cesium-137, strontium-90, and cobalt-60 show no definite correlation with potassium, calcium, and cobalt, respectively, indicating that these elements are in large measure supplied from imported foods.

With the current means of sanitation—pit toilets and burial of garbage—on Rongelap and Eniaetok Islets there must be a net addition of minerals. The chief export, copra, is low in ash content as compared with imported foods. A quantitative evaluation of the addition would require comparison of export and import records.

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The Protein Quality, Digestibility, and Composition of Algae, Chlorella 71105[a]

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SUMMARY

Freeze-dried *Chlorella* 71105 was found to contain 55.5% crude protein; 7.5% crude fat; 8.25% ash; 7.0% moisture; 17.8% total carbohydrate; 3.1% crude fiber; 2.68% chlorophyll; and .08% urea. The digestibility of the protein was 86%; of crude fat, 93%; of total carbohydrate, 72%; and of crude fiber, 15%. The protein efficiency ratio (PER) was 2.19, which compares favorably with soy protein. The PER of *Chlorella* with 0.2% L-methionine was 2.90, which compares favorably with casein values in the literature. The PER of casein was 3.30; that of defatted egg protein, 4.01, in these studies. The rats were fed at 10% protein levels. In addition, *Chlorella* 71105 contained all essential amino acids as well as having a high carotene content. Available energy value was 3.3 kcal/g.

For extended exploration into space, a system must be designed to provide such astronaut needs as air revitalization, waste disposal, water recovery, and food supply. From a logistic standpoint, algae would provide an ideal gas-exchange medium because of their capability to convert CO_2 into cellular constituents while producing O_2. Furthermore, treated waste material can provide mineral nutrients and water to support algal growth.

Before algae can be accepted as human food, the nutritional characteristics of the product, grown by mass-culture techniques, must be evaluated. The Sorokin-Myers strain of *Chlorella* 71105 is currently under extensive research in connection with photosynthetic gas exchange. Because nutritional studies with this strain have been incomplete, the following studies were undertaken: 1) composition, i.e., proximate analysis, vitamins, minerals, and amino acids; 2) digestibility of protein, fat, carbohydrate, and crude fiber; 3) quality of protein; 4) caloric value.

[a] The work reported was carried out under Contract No. AF 33(616)-7373, Biomedical Laboratory, Aerospace Medical Laboratory, U. S. Air Force, Wright-Patterson Air Force Base, Ohio.

LITERATURE REVIEW

Fisher and Burlew (Burlew, 1953) reported an average protein (crude) content of 50% in the dried green algae *Chlorella pyrenoidosa,* compared with 60% for fish meal, 48% for dried yeast, 44% for soybean meal, and 36% for dried skim milk. Those authors also gave vitamin assays for dried *Chlorella pyrenoidosa.* Milner (1948) reported striking variations in the lipid content of *Chlorella pyrenoidosa,* from 5 to 85% of the dry weight, depending on culture conditions. Values of 4–6% chlorophyll in *Chlorella* were reported by Fisher (Burlew, 1953). Powell *et al.* (1961) reported 59% protein, 19% fat, 13% carbohydrate, 3% moisture, and 6% ash in a vacuum-dried mixture of *Scenedesmus obliquus* and *Chlorella ellipsoida.*

Morimura and Tamiya (1954) reported 200–500 mg% of ascorbic acid in *Chlorella ellipsoida,* and Cook (1960) reported 39.6 mg% ascorbic acid and 55.4 mg/100 g of β-carotene in dried algae (a mixture of *Scenedesmus* and *Chlorella*) grown in sewage.

Fisher and Burlew (Burlew, 1953) reported methionine values of 0.36 and 0.57%

for dried *Chlorella* of somewhat lower protein content than that used in this study.

Prosky and Karinen (1960) reported a digestibility of 70% for the protein of algae (*Scenedesmus* and *Chlorella*) supplied by the Japan Nutrition Association. Cook (1960) reported 54% digestibility for the protein of sewage-grown algae consisting of *Scenedesmus* and *Chlorella*. Hayami and Shino (1958) reported 60% digestibility for dry *Chlorella*.

Geoghegan (Burlew, 1953) reported a protein efficiency ratio (PER) of 1.84 for *Chlorella*, and Cook (1960) and Cook and Lau (1961) reported a PER of 1.62 for a dried waste-grown mixture of *Scenedesmus* and *Chlorella* algae, which became 1.85 after cooking. Powell *et al.* (1961) reported 5.5 kcal/g (bomb calorimeter) for dried algae supplied by the Japan Nutrition Association. They also fed this algae (a mixture of *Chlorella* and *Scenedesmus*) to human volunteers. Mild to severe gastrointestinal symptoms of a transient nature resulted.

MATERIALS AND METHODS

The composite algae powder was prepared from a culture of *Chlorella pyrenoidosa* 71105 grown in open conditions within an air-conditioned facility by sanitary food-plant procedures. The *Chlorella* was grown continuously at 39°C in two 600-gal. stainless-steel tanks. Fluorescent lamps were used as the light source, and 0.7% carbon dioxide in filtered air was constantly bubbled through the standard medium. The water used in the culture medium was filtered through acetate-wound filters. The algae were harvested in a Sharples super-centrifugal separator. The *Chlorella* paste was then spread on stainless-steel trays and frozen at −10°C. These trays were placed in a vacuum dryer. After about seven hours the trays and dried *Chlorella* were removed. The dried material was milled to a powder and blended with other *Chlorella* prepared in the same manner to give a composite sample of sufficient size to conduct the study program.

Vitamin assays were determined as follows: ascorbic acid, dye titration method (Schaffert and Kingsley, 1955); B_{12}, yeast assay with *S. carlsbergensis* (Atkin *et al.*, 1943); thiamine, thiochrome method (AOAC, 1960); pantothenic acid, microbiological assay (Novelli *et al.*, 1951); and β-carotene by the chromatographic method (AOAC, 1960). Amino acids were determined by the methods of Spackman *et al.* (1958).

To determine protein quality, four groups of ten male litter-mate weanling rats were fed isocaloric rations, each containing 10% available protein. The rations contained one of the following as the sole source of protein: *Chlorella; Chlorella* supplemented with L-methionine; purified casein; and defatted egg protein (see Table 1).

Table 1. Diets used for protein quality studies.[a]

	Chlorella (%)	Casein (%)	*Chlorella* plus L-methionine (%)	Defatted egg protein (%)
Chlorella 71105 Lot A926-125	21	.....	20.5	.....
Casein [b]	.....	12.3	.....	.....
L-Methionine	.....	.....	0.20	.....
Dried defatted egg powder [c]	.....	.....	.....	11.7
Corn oil	10	9	10	9
Starch	61	70.7	61.3	66.3
Agar	2	2	2	7
Vitamins in dextrose [d]	.....	2	.....	2
Vitamins in dextrose without vitamin A [d]	2	.....	2	.....
Salt mixture (USP XV)	4	4	4	4
Total	100	100	100	100
Kilocalories per 100 g	403	404	404	403

[a] All diets contain 10% available protein based on nitrogen × 6.25 and digestibility of protein.

[b] Casein (purified) was purchased from Nutritional Biochemicals Corporation, Cleveland 28, Ohio.

[c] Dried defatted whole egg protein powder was purchased from Wilson Laboratories, 4221 Southwestern Boulevard, Chicago 9, Illinois.

[d] Vitamin diet fortification mixture in dextrose, with and without vitamin A, was purchased from Nutritional Biochemicals Corporation, Cleveland 28, Ohio.

Table 2. Analysis and vitamin content of dried powdered *Chlorella*.

Protein (nitrogen × 6.25)	55.5%
Crude fat	7.5%
Carbohydrate, total (anthrone method)	17.8%
Ash	8.25%
Moisture (hot-air oven at 105°C)	7.00%
Crude fiber (cellulose and hemicellulose)	3.1%
Urea[a]	0.08%
Chlorophyll	2.68%
Total calories	5.2 kcal/g
Ascorbic acid	14.6 mg/100 g
Vitamin B_6	3.0 μg/g
Thiamine (B_1)	7.7 μg/g
Pantothenic acid	11.2 μg/g
β-Carotene	50.2 mg/100 g

[a] Urea was carried over from the growth medium.

The experiment was allowed to run for 28 days. Records were kept of food consumption and food wastage. The rats were fed *ad libitum* and were weighed periodically. Vitamin A was omitted from the *Chlorella*-containing rations because of the substantial amounts of β-carotene already present. PER is the ratio between the weight gained and the weight of protein consumed.

The caloric value of *Chlorella* was determined by calculation using proximate analysis with corrections for digestibility and urinary energy loss per gram of protein. The caloric value was also determined using the bomb calorimeter.

RESULTS

Composition. Table 2 shows the analysis of dried *Chlorella*. The crude fat in *Chlorella* was liquid, and Milner (1948) reported that it was unsaturated.

Semiquantitative spectrographic analysis was carried out on *Chlorella*. Percentages were: phosphorous, 1–10%; calcium and magnesium, 0.1–1%; iron and silicon, 0.01–0.1%; sodium, aluminum, barium, and copper, 0.001–0.01%; bismuth, chromium, lead, manganese, and titanium, less than 0.001%. Table 2 also shows the determinations of ascorbic acid, β-carotene, B_6, thiamine, and pantothenic acid. Riboflavin and niacin were not determined in this study, but this should be done in the future.

Table 3 shows the amino acid content of *Chlorella*. All of the essential amino acids were present.

Digestibility. The coefficients of digestibility were determined for the protein, fat, total carbohydrate, and crude fiber content in this strain of *Chlorella*. The digestibility was 86% for protein, 93% for crude fat, 72% for total carbohydrate, and 15% for crude fiber.

Protein quality. Protein efficiency ratio (PER) experiments were carried out to compare the quality of algae protein with other food proteins. The PER of *Chlorella* was 2.19, *Chlorella*-methionine 2.90, casein 3.30, and egg protein 4.01.

Caloric value. The caloric value of *Chlorella* was determined both by calculation and by bomb calorimetry. The values were 5.16 kcal/g by bomb calorimetry techniques, and 3.29 kcal/g by calculation.

DISCUSSION

Chlorella contained more crude protein than dried beef, soybean meal, dried yeast, or skim milk powder, and contained significant amounts of calcium, phosphorous, and iron. It can also be considered an excellent source of β-carotene and a less significant one of ascorbic acid. Values for carotene in *Chlorella* were significantly greater than those reported for alfalfa leaf meals.

Protein with an amino acid pattern such as that contained in *Chlorella* should be suitable as supplemental food. Our methionine value is substantially greater than values reported in the literature. The digestibility of the protein (86%, higher than the 54–70% found in the literature) compares favorably with values for beef liver, fish meal, and meat scraps.

Table 3. Amino acid content of powdered *Chlorella*.

	Amino acid / nitrogen (g/g)
Aspartic acid	0.37
Threonine	0.20
Serine	0.14
Proline	0.25
Glutamic acid	0.58
Glycine	0.30
Alanine	0.37
Valine	0.32
Methionine	0.11
Isoleucine	0.21
Leucine	0.25
Tyrosine	0.17
Phenylalanine	0.28
Lysine	0.49
Histidine	0.09
Arginine	0.35
Tryptophan	0.09

The PER was higher for *Chlorella* than for most vegetable and cereal proteins, and slightly higher than for soy protein. The PER of *Chlorella* plus methionine, although lower than our own value for casein, compares favorably with literature values for casein or milk protein. It is about the same as for beef liver and hamburger.

Our values for egg protein and casein are higher than most literature values (Wrenshall, 1960; Burlew, 1953; Cook, 1960; Cook and Lau, 1961). Using three different strains of rats, Campbell (1960) found PER values of 2.59, 3.18, and 3.35 for casein, and 3.44, 4.00, and 4.00 for dried whole egg. He concluded that rat strain is the most important factor in variations of PER values among laboratories. He proposed the following correction calculation:

$$\text{PER} \times \frac{2.5}{\text{PER for casein}}$$

This calculation assumes a PER of 2.5 for reference-standard casein and corrects for variation due to strain of rat. Using Campbell's correction calculation, we found the following PER values: casein 2.5, egg protein 3.03, *Chlorella*-methionine 2.20, *Chlorella* 1.66. Since most investigators have not used Campbell's method of correction for strain of rats, the errors inherent in all comparisons of PER are self-evident.

More work, using higher levels of L-methionine for *Chlorella* supplementation to bring levels up to: 1) that contained in egg, and 2) that believed to cause maximum growth in rats, should prove rewarding.

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Food Crushing Sounds. An Introductory Study[a,b]

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SUMMARY

The sounds produced by chewing foodstuffs, or by crushing them between flat surfaces, were tape recorded and analyzed as to amplitude, frequency, and duration. Preliminary data are given on chewing sound characteristics, including certain differences between foodstuffs.

An appreciable part of the literature of food rheology is devoted to the measurement of the slowly varying forces necessary to crush foodstuffs (Kramer and Twigg, 1959). Biting forces (Howell and Manly, 1948; Potter *et al.*, 1947), jaw movements during chewing (Klatsky, 1940; Klatsky and Fisher, 1953), and relations between jaw positions and various aspects of hearing (von Békésy, 1932, 1939; Franke *et al.*, 1952) have also been studied. However, only little attention has been given to vibrations produced by the action of the complicated feedback mechanism that regulates the rate and force and duration of chewing.

The possibility exists that physical measurements of crushing sounds could be helpful in the field of food texture by providing new information to correlate with sensory data. As an introduction to such work, this paper is concerned with the development of methodology and with a study of relevant variables.

METHODS

Apparatus. The equipment consisted of the following components, which were used to obtain strip-chart recordings of sound spectra and of amplitude variations with time.

For sensing of sounds. The sound pick-up device was a dynamic microphone of a low-impedance directional type (Signal Corps, U. S. Army, M-44A/U) or a hearing-aid earphone of a high-impedance extended-range type (Dyna-Lab, similar to Danavox DT). In an attempt to isolate the body of the earphone from vibrational contact with the head of the chewing subject, it was connected to the ear canal via a short plastic tubing.

For tape recording and reproduction of sounds. The recorders were: 1) a sound recorder-reproducer set for 2400 feet of ¼-inch half-track magnetic tape with speeds of 15 and 7½ inches per second, and provided with a spring-loaded pulley so that endless tape loops could be used instead of conventional continuous (nonloop) tape; 2) a less versatile sound recorder-reproducer with speeds of 7½ and 3¾ inches per second. (Signal Corps, U. S. Army, respectively, AN/TNH-2A and RD-87A/U for 1) and 2); both manufactured by Telectro Industries Corp., Long Island City, N. Y.)

For frequency analysis. Used for frequency analysis was the audio spectrometer part (Model No. 2109) of an automatic spectrum recorder with 31 channels, of which 27 gave 1/3-octave bands for the range 40–16000 c/s, 3 gave weighted responses corresponding to standards for objective sound-level meters, and one gave a linear response corresponding to the total sound (Brüel & Kjaer, Copenhagen, Denmark, Model 2311).

For strip-chart recording of amplitude data. Used for strip-chart recording were: 1) a vacuum-tube voltmeter with instrument terminals

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[b] Presented at the 22nd annual meeting of the Institute of Food Technologists, Miami Beach, June, 1962, as "Analysis of Mastication Sounds."

[c] Visiting scientist from the Swedish Institute for Food Preservation Research, Göteborg 16, Sweden.

carrying a DC component (Voltohmyst WV-87B or WV-77E). 2) An attenuator consisting of a decade resistance box in series with two resistors of 33 and 6.8 kilo-ohms. The output to the strip-chart recorder was the terminals of the box, which was usually positioned at values ranging from 300 to 3000 ohms. The series combination of the box and the 6.8 kilo-ohms resistor could, when desired, be shunted with a 100-microfarad electrolytic capacitor. 3) A strip-chart recorder for 1 mV DC with 1-second full-scale pen travel (Model 143X58, Electronik, Minneapolis-Honeywell Regulator Co.).

For auxiliary purposes. Also used were: 1) a dual-beam cathode-ray oscillograph (Type 322-A, Du Mont), which was usually connected to the input and the output of the frequency analyzer; and 2) an audio generator (Sine-Square Wave Generator, Model AG-10, Heathkit).

Foodstuffs. During initial stages cookies, crackers, potato chips, apples, and chewing gums were used. Later work was performed mostly with crisp bread (Swedish-style "Knäckebröd," brown, and "Delikatess," white), beef, apple, crisp head lettuce, and peanuts. Tenderness-rated dehydrated beef had been prepared at the Institute. The other foodstuffs were commercial products.

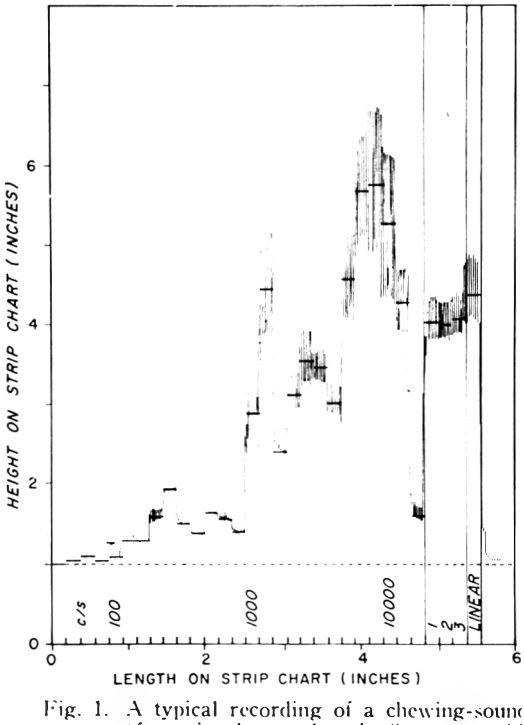

Fig. 1. A typical recording of a chewing-sound spectrum (for crisp brown bread; "open mouth" technique) with visually estimated mean values marked as horizontal lines. Along the abscissa, from left to right: 27 frequency bands, centered on frequencies from 40 to 16000 c/s; 3 weighted responses; one linear response.

Techniques. There are, principally, two different ways of using a tape recorder in combination with a frequency analyzer. One is to use an endless loop of tape, so that the playback of a short recording can be frequently repeated while the frequency analyzer sweeps over a series of bands, and the other is to run the recorder in the regular (nonloop) way. In the first case, the strip-chart record gives a sound-amplitude spectrum that, with a frequency analyzer having discrete bands, will appear step-shaped (*cf.* Fig. 1). In the latter case, a continuous strip-chart recording has to be made for every frequency band of interest (*cf.* Fig. 11). The first method gives "time average" data, whereas the latter gives "time course" data.

Application of sensing device. While the subjects chewed a piece of food, the mastication sounds were taken off by one of the following methods: a) The microphone was pressed against the cheek on the side where chewing was done; b) the microphone was held 2 inches in front of the open mouth, while the subject chewed between the front teeth, or c) the plug of the earphone was inserted into one ear canal in such a way as to close the orifice to the outer air, while the subject chewed on the side where the earphone was held. In some cases, foodstuffs were also pressed between a block of wood and the surface of a table, while the microphone was held 2 inches from the edge of the block. The four methods are respectively referred to herein as "through cheek," "open mouth," "ear canal," and "wood block."

Recording of sounds. When recording on tape loops, 15 inches per second was the usual speed, and the length of the loops was 30 inches, i.e., 2 seconds. The erasing head of the recorder AN/TNH-2A was often disconnected during work with tape loops. This gave a better utilization of the magnetic layer, while at the same time the biasing AC voltage on the recording head was sufficient to give enough erasing action to prevent a serious build-up of several recordings on top of each other. The start and stop of a recording on a tape loop was usually effected by manually closing and opening the shutter that presses the tape against the recorder head. This procedure gave recordings without switch-off clicks, which in preliminary experiments showed up as disturbances in the spectra. To obtain a stable recording level, a constant tone was recorded on available brands of magnetic tape, and those found most homogeneous were selected. Care was taken not to overload the electronic amplifiers. This was especially important since the impact-type sounds of chewing do not ordinarily

show up well on the moving-coil instruments of the recorder-reproducer and of the VTVM. All recordings were made in a quiet, but not soundproof, room.

Recordings were usually started with the second or third bite. This was adopted to avoid erratic results from irregular starts of the chewing.

Reproduction of tape records ("playback"). The low-impedance output from the recorder-reproducer was connected through a shielded cable to the amplifier input of the frequency analyzer. For work with tape loops, the latter was run with the automatic sweep, ordinarily set to give a rate of about 8 minutes for all 31 positions. For work with continuous (nonloop) tape, the sweep was, of course, not used. Strip-chart recordings from tape loops were usually made with a paper speed of ⅔ or 2 inches per minute, whereas for continuous tape 2 or 8 inches per minute was used.

In some cases, a tape record obtained at a speed of 15 inches per second was played back at 3¾ inches per second and simultaneously recorded on the other recorder at 15 inches per second. When the new record was played back at 3¾ inches per second to the frequency analyzer, the strip-chart recording was extended 16 times. This procedure gave a better resolution along the time axis, but, of course, some information was lost since the lower part of the original spectrum fell outside the range of the available equipment.

Evaluation of strip-chart recordings. The ordinary way of treating strip-chart recordings was to copy the visually estimated mean for each one of the frequency bands on another paper. The heights were then measured in millimeters and the measured values were transferred to semilogarithmic diagrams. Fig. 1 shows an example of a strip chart recording with mean values marked off.

For continuous-tape (nonloop) records, the strip-chart recordings were either copied and compared visually, or the heights of the peaks were measured, tabulated, and statistically evaluated.

Controls. Three repeated strip-chart recordings of the same tape loop record gave the data in Fig. 2-A, and single strip-chart recordings of three different tape-loop records for the same foodstuff gave the data in Fig. 2-B.

Chattering sounds, produced by rapidly closing the jaws at a steady rate without anything between the teeth, were of much shorter duration than sounds obtained with food. The coefficient of variation (standard deviation divided by the mean) for 35 consecutive peaks was 6% for the total sound and 38%, 28%, and 29%, respectively, for the frequency bands 200, 630, and 2000 c/s.

MEASUREMENTS

Time average experiments. The technique with tape loops was used to obtain information about mastication and crushing-sound characteristics for a number of foodstuffs.

Comparisons between various foodstuffs. At an early stage of this work, a series of foodstuffs were crushed, and the sounds produced were recorded by the following techniques: a) "through cheek," b) "open mouth," and c) "wood block." These experiments gave the data in Fig. 3, in which the points indicate mean values for, usually, 2 or 3 chewings by one person, or 2 or 3 crushings. To take out some of the step-function effect of the frequency bands, all values in Fig. 3 have been smoothed by weighting with ¼ of the two adjacent values.

Comparisons between people. Two series of experiments were performed to estimate the variation of chewing sounds between people.

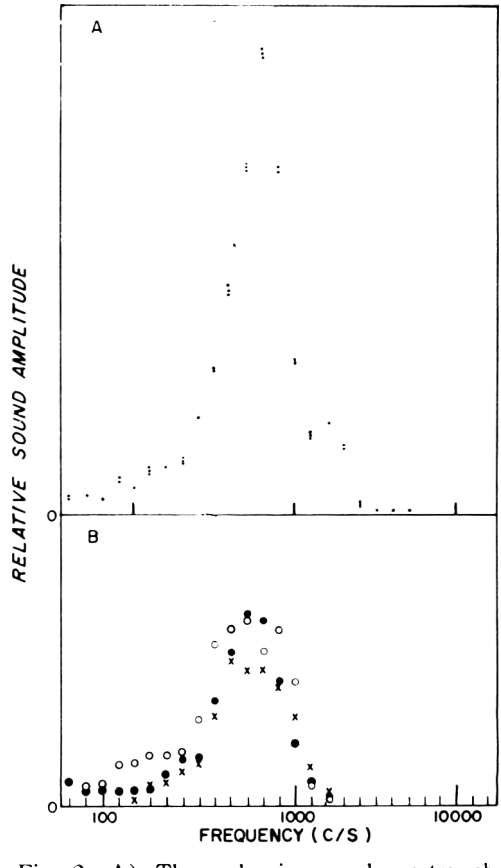

Fig. 2. A) Three chewing-sound spectra obtained from the same tape record (apple, "through cheek" technique); B) three chewing-sound spectra obtained from separate tape records (cookie, "through cheek" technique).

In the first series, crisp brown bread was chewed with the mouth open, 4 times by each of 5 persons, and sound spectra were obtained for the region 800–16000 c/s. Fig. 4 shows that peaks occurred at approximately the same frequencies

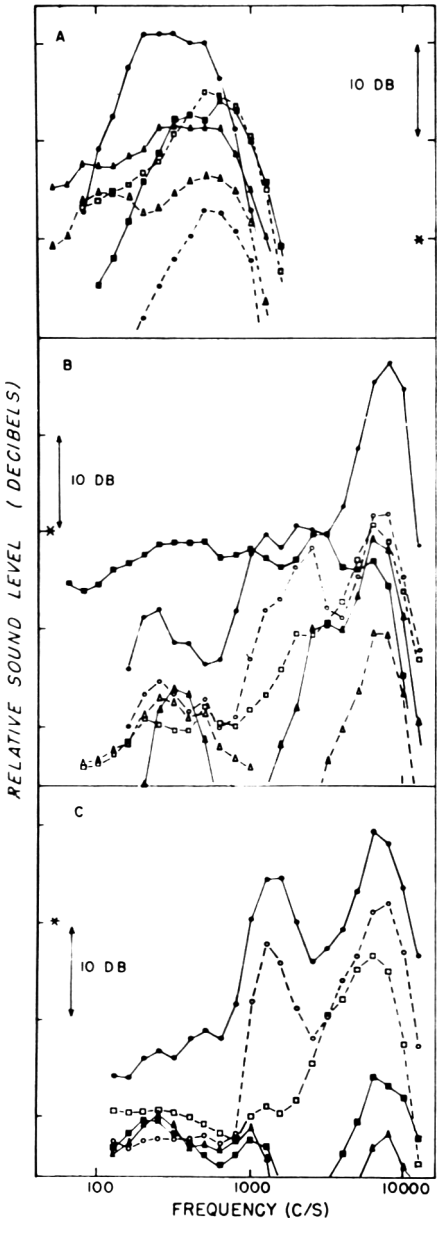

Fig. 3. Chewing-sound spectra obtained for six foodstuffs with the following techniques: A) "through cheek"; B) "open mouth"; and C) "wood block." ○ = crisp white bread; □ = crisp head lettuce; △ = ham; ● = crisp brown bread; ■ = apple; ▲ = sausage. A sound level of 20 db above a "general noise level" is indicated by an asterisk.

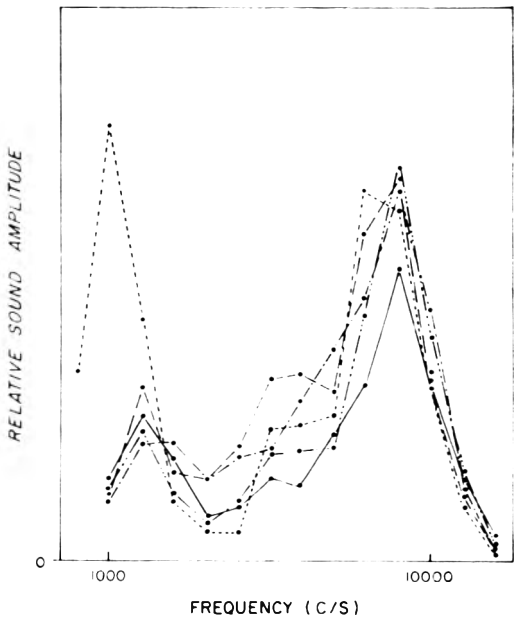

Fig. 4. Sound spectra for 5 persons chewing crisp brown bread ("open mouth" technique). Each point indicates the mean amplitude for four samples.

and had approximately the same heights. However, the dispersion is greater in the region 2000–6300 c/s than at the two peaks at 1250 and 8000 c/s. Also, there is an anomaly at 1000 c/s, where one person showed exceptionally high values (unfortunately, this person was not available for a recheck). The standard deviation of amplitude values was relatively independent of frequencies and persons. The coefficient of variation for the peak values in the vicinity of 10,000 c/s varied between 13 and 31%. An analysis of variance demonstrated a significant difference ($p < 0.05$) between people at 6300 c/s, but not at the frequencies 5000, 8000, and 10000 c/s. The amplitudes for the other frequencies were not considered large enough to warrant a finer analysis.

In the second series, each of 4 persons chewed 4 samples of tough and 4 samples of tender beef, using the "ear canal" technique. Pooled data for each person are plotted in Fig. 5 in the form of a partial spectrum.

Effect of toasting. To obtain a series of foodstuffs differing in only one relevant aspect, soft white bread was toasted to various degrees, and samples were chewed with the "open month" technique. This gave spectra of the type shown in Fig. 6. A plotting of peak amplitude values for two persons against degree of browning (measured by a subjective scale) gave the data in Fig. 7.

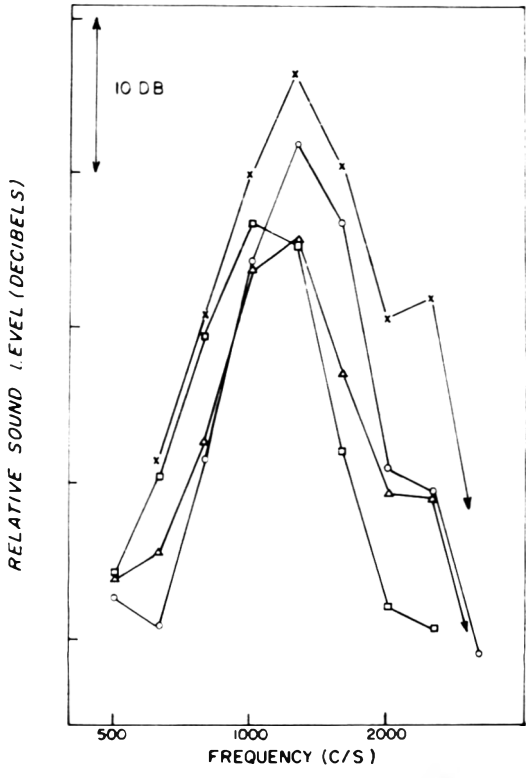

Fig. 5. Spectra for sounds produced by 4 persons chewing beef ("ear canal" technique; mean values of 8 experiments each).

Relation of sound level to tenderness-toughness. Each of 4 persons chewed 4 samples of tender and 4 samples of tough beef, whose ratings on a 9-point unstructured scale (Raffensperger *et al.*, 1956) were 7.5 and 4.5, respectively. Fig. 8 shows a plot of mean sound level for all 16 determinations for each one of the two foodstuffs (*cf.* Fig. 5, which is based on the same original data).

The mean values for the difference between sound level for tough and for tender meat in the frequency range 800–2500 c/s for the four persons were 1.6, 2.0, 3.2, and 3.5 decibels. Thus, sound level was significantly higher ($p < 0.02$) with tough meat than with tender meat. The combined mean difference was 2.6 decibels, i.e., the intensity of mastication sounds was 35% higher with tough meat than with tender meat.

Time-course experiments. The continuous-tape (nonloop) technique was used to obtain information about the time course of chewing sounds over more extended periods than the 2 seconds covered by the tape-loop technique.

Regularity of chewing. Fig. 9 shows number of bites as a function of time for two experiments with two quite different foodstuffs: crisp brown bread and crisp head lettuce. The common slope of the two lines in Fig. 9 corresponds to a rate of approximately 1.85 bites per second. In other experiments, the values ranged from 1.5 to 1.9 (*cf.* Fig. 11).

General decline in sound level. During normal chewing, the structure of a foodstuff is gradually broken down until the "chew" is ready for swallowing. Such a breakdown can be expected to be paralleled by a decline in the average amplitude of successive bursts of mastication sounds. This factor was studied by chewing various types of food for up to 30 seconds.

Pieces of crisp brown bread, peanuts and half peanuts, small cubes of apple, and pieces of soft white bread were chewed, and the sounds were recorded at a tape speed of 15 inches per second with the microphone pressed to the cheek. During playback at 7½ inches per second, the strip chart was run at a speed of 8 inches per minute. This enabled a measuring of peak amplitudes for the "biting" and "opening" sounds separately while not giving enough resolution to determine the precise shape of the peaks (*cf.* below). Pooled data for biting sounds in three experiments each with three foodstuffs are plotted in Fig. 10. For soft bread, the values usually fell within the region

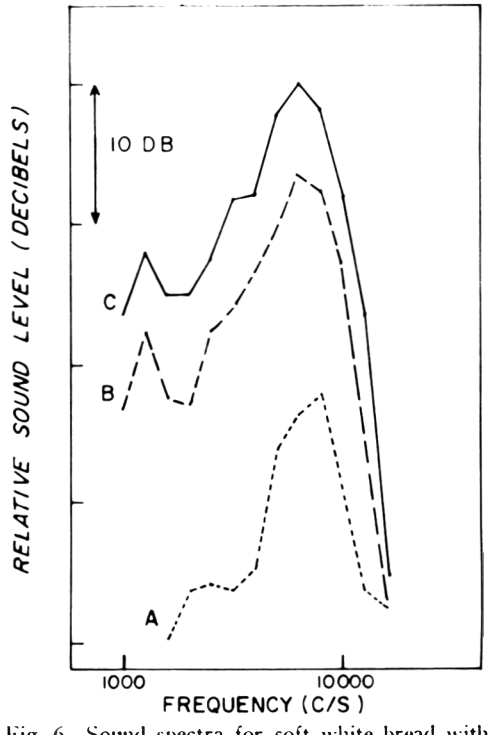

Fig. 6. Sound spectra for soft white bread with various degrees of toasting ("open mouth" technique). A) not toasted, B) light-toasted, and C) hard-toasted.

of general noise, and only a simple calculation of the mean amplitude was performed.

A calculation of regression lines, fitted to the data by the method of least squares, indicated that the first part of the curves for crisp brown bread and for a half peanut could best be represented by the left half of a parabola, whereas a straight line was most appropriate for the apple (*cf.* solid lines in Fig. 10). The actual regression equations were the following (A = amplitude on an arbitrary scale, and t = time in seconds):

Crisp brown bread	$A = 84.5 - 8.78t + 0.33t^2$
Half peanut	$A = 60.1 - 6.54t + 0.256t^2$
Apple	$A = 22.1 - 0.53t$
Soft white bread	$A = 10.6$

The coefficient of the first degree term for crisp brown bread and for half peanuts was significantly different from zero ($p < 0.001$), whereas for apple the coefficient was not significantly different from zero ($p > 0.05$). As determined from these coefficients, the initial average rate of decrease in amplitude was approximately 5.7% per bite for crisp brown bread and for half peanuts.

Fig. 10 shows that a general noise level is reached for various foodstuffs after not more than 15 seconds, corresponding to approximately 20 bites.

Comparison between biting and jaw separation sounds. Some of the tape records used for general decline determinations were re-recorded by the speed-changing playback-procedure described above. The new tape records were then played back without using the electrolytic condenser in the attenuator circuit. This gave strip-chart recordings that were extended enough to permit the plotting of individual curves for sounds produced

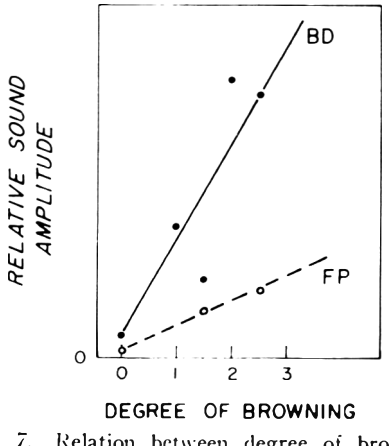

Fig. 7. Relation between degree of browning and peak amplitude of sounds produced by two persons chewing toasts ("open mouth" technique). The scale on the abscissa indicates: 0 = white, 1 = yellow, 2 = brown, and 3 = dark brown.

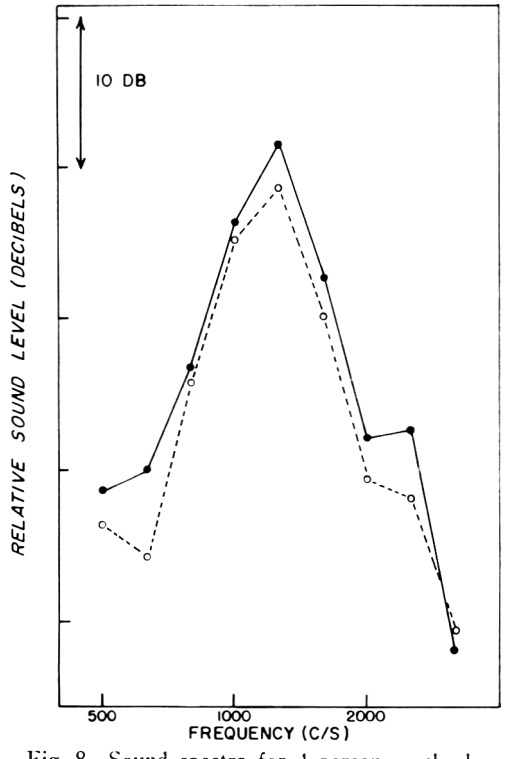

Fig. 8. Sound spectra for 4 persons each chewing 4 samples of tough (●) and 4 samples of tender (○) beef.

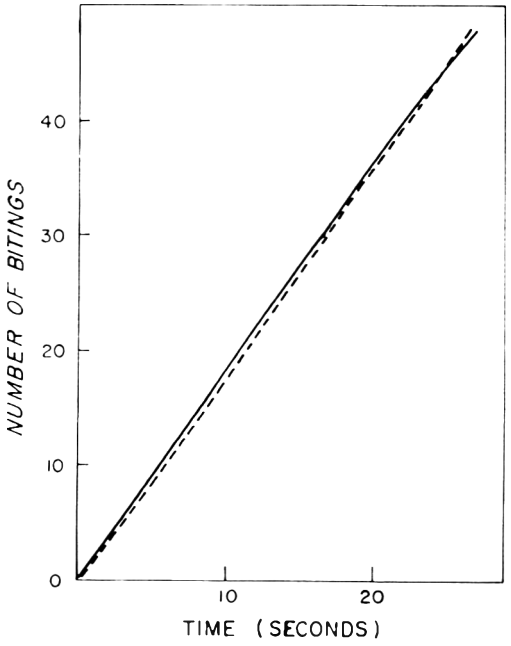

Fig. 9. Number of bites as a function of the time for one person chewing crisp brown bread (– – – –) and crisp head lettuce (——).

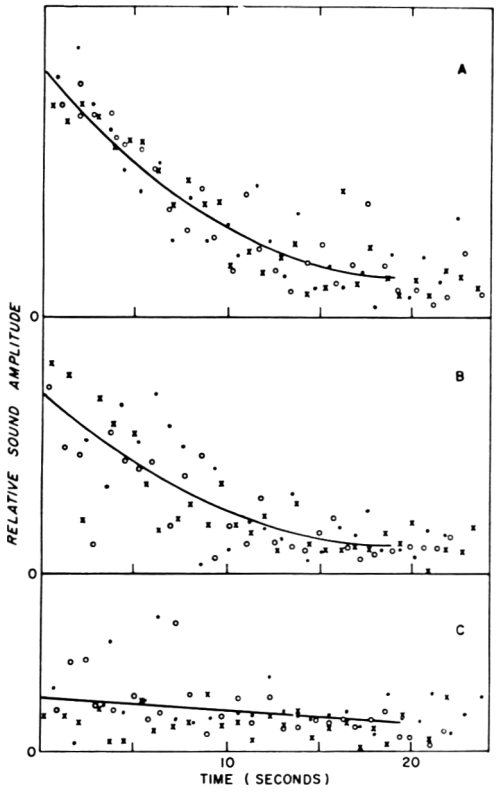

Fig. 10. General decline in mastication sound amplitude for A) crisp brown bread, B) a half peanut, and C) apple. The three types of symbols relate to three different experiments on each foodstuff.

during biting, and for sounds produced during separation of the jaws.

Fig. 11 shows examples of such curves obtained at 50 c/s, which corresponds to an original frequency of 800 c/s, and Fig. 12 shows a visually averaged picture for 12 separate curves.

DISCUSSION

Because of the dampening action of the cheeks and tongue, it can be assumed that practically all vibrations, which are perceived as chewing sounds, reach the inner ear by bone conduction. Thus, the resonance properties of the skull (von Békésy, 1932) tend to equalize the responses for different foods (*cf.* Fig. 3-A). When there is no dampening by soft tissues, however, the differences in crushing sounds between foodstuffs come out larger (*cf.* Fig. 3-B,C).

It can further be assumed that chewing involves a recurrent alternation between a build-up of static forces, when the food is clamped between the teeth, and a burst-out of vibrations, when it is fractured. Thus, it is likely that mastication sounds and related vibrations are utilized as a complement to static clues in subjective estimation of the mechanical properties of food. Such a concept is in accordance with recent work on the dynamic behavior of the sense of touch (Eijkman and Vendrik, 1960), but it would contradict a strict adherence to the opinion that chewing sounds constitute an objectionable noise (Bárány, 1938).

The recording of sounds through the cheek with a commercial microphone probably involves some "seismograph" and/or "accelerometer" action. Thus, this technique may give an empirical measure of differences between foodstuffs, but cannot give easily interpreted information about the actual vibrations of the foodstuffs and of the jaws. The "open mouth" procedure, which can be calibrated by standard methods, does not necessarily give a true picture of normal chewing, in which the vibrations are dampened by contact of the foodstuffs with the soft tissues inside the mouth. The "wood

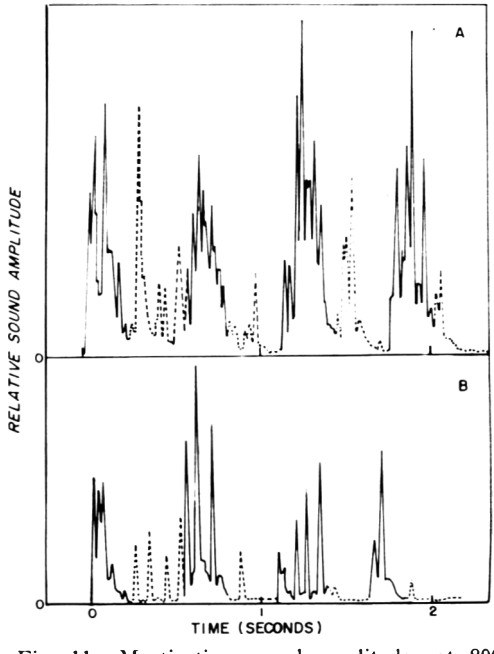

Fig. 11. Mastication sound amplitudes at 800 c/s as a function of time for A) crisp brown bread and B) a half peanut. Solid lines = "biting" sounds; dashed lines = "opening" sounds.

block" and the "open mouth" techniques seem to give similar results, but a final evaluation of the degree of similarity has to await further comparisons. Of course, correlations between the "wood block" technique and other types of rheological measurements would also be useful. The use of an earphone as a pick-up device for airborne sound in the ear canal may give a true picture of bone-conducted sounds, but the possible interference between chewing movements in general and specific food-crushing sounds needs to be explored further before the merits of this method can be established.

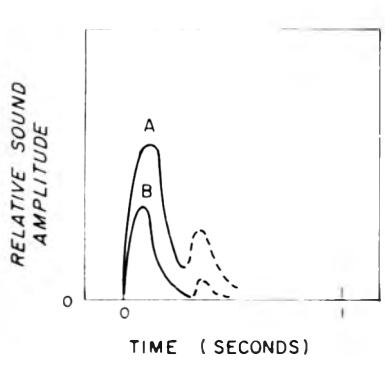

Fig. 12. Visually averaged curves for 12 chews of each of two foodstuffs (for explanations, see Fig. 11).

An introspection showed that the "biting" sounds were produced mainly by the crushing action itself, whereas the "opening" sounds originated both in a reforming of the "chew" and in movements of tongue, teeth, saliva, etc. The "opening" sounds were commonly found for softer foodstuffs in a broad region around 160 c/s (*cf.* Fig. 3-A, ham and sausage). Unfortunately, however, when lower frequencies were reproduced at a very low tape speed, they fell outside the useful range of the equipment, and, consequently, the procedure used to obtain Figs. 11 and 12 could not be used for investigating the time course of such sounds.

One way of increasing the resolving power of the physical analysis described here would be to supplement it with other techniques such as that of "visible speech" (Potter *et al.*, 1947). Unfortunately, such equipment was not available during this exploratory work.

Harrington and Pearson (1962) have found that approximately 25–47 bites are commonly made before pork meat is swallowed. These values are much higher than the highest values at which the mastication sound level decreases to a general noise level (*cf.* Fig. 10 and related text). This suggests the possibility of dividing the normal chewing into two phases, one involving a gross cutting and the other a finer comminution.

Some of the chewing-sound differences between people are appreciable in comparison to the corresponding differences between foodstuffs (*cf.* Figs. 5 and 8), whereas in other cases they are relatively unimportant (*cf.* Figs. 3-B and 4). The data reported here may serve as a starting point for future investigations, which could aim at a careful analysis of, e.g., the statistical variation of sound level within single bites (*cf.* Fig. 11) and other qualitative differences between related foodstuffs, as compared to the mainly quantitative differences reported in Figs. 6 and 8. Also, this type of recording and analysis may prove to be a useful tool in the study of interpersonal differences in chewing behavior. Before chewing-sound data can be utilized in establishing analytical procedures for food testing, however, experiments must be performed and statistically evaluated with many foods and many people. Work in this direction has been planned.

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