Journal of
FOOD SCIENCE
(formerly Food Research)

176 West Adams Street
Chicago, Illinois 60603

VOLUME 29

JANUARY-DECEMBER, 1964

Printed by
THE GARRARD PRESS
CHAMPAIGN, ILLINOIS

Journal of
FOOD SCIENCE

SUBSCRIPTION RATES

To members of the Institute of Food Technologists; Domestic, $7.50 per year; Canada, Mexico, Central and South America, plus $1.00 postage; all other countries, plus $2.00 postage.

Non-Member Subscription Rates: Domestic, $8.50 per year: Canada, Mexico, Central and South America $10.00 per year plus $1.00 postage, all other Countries $10.00 per year plus $2.00 postage; Single copies, Domestic, $1.50 each; Foreign, $1.75 each.

All subscriptions are entered to begin with the first issue of the current volume. One volume per year published starting with the January-February issue. Subscription orders received after February 15 will be pro-rated for the balance of the current volume. Issues published prior to date of receipt of subscription order will be figured at the single copy price if available.

Copies of many back numbers are available at the single copy rate. Remittance to accompany order.

CORRESPONDENCE

Editorial: DR. G. F. STEWART, Executive Editor, P.O. Box 98, Davis, California.

Non-Member Subscriptions, Back Volumes, Single Copies, and Reprints: JOURNAL OF FOOD SCIENCE, 510-524 N. Hickory Street, Champaign, Ill.

Member Subscriptions: THE INSTITUTE OF FOOD TECHNOLOGISTS, 176 W. Adams St., Chicago 3, Ill.

Advertising: CALVERT L. WILLEY, Business Manager, 176 W. Adams St., Chicago, Ill.

CLAIMS

Claims for copies lost in the mails must be received within 30 days (90 days foreign) of the date of issue. Notice of change of address must be received two weeks before date of issue.

NOTE

The Institute of Food Technologists assumes no responsibility for statements in articles appearing in JOURNAL OF FOOD SCIENCE. The opinions expressed do not necessarily reflect policies of the Institute of Food Technologists.

Manuscripts for JOURNAL OF FOOD SCIENCE should conform to the style used in the journal and be submitted in duplicate to the Executive Editor. Authors of papers intended for JOURNAL OF FOOD SCIENCE should request from the Editorial office a copy of the booklet "Research papers for IFT ournals." JOURNAL OF FOOD SCIENCE reserves the privilege of editing manuscripts to make them conform with the adopted style of the journal or returning them to the author for revision. Changes made by the Editorial Office may be reviewed by authors when they examine proof.

Published by the Institute of Food Technologists six times a year. Printed by The Garrard Press, 510-524 N. Hickory Street, Champaign, Illinois. Entered as second class matter at the Post Office at Champaign, Illinois, under the act of March 3, 1879. Accepted for mailing at special rate of postage provided for in amended Section 34.40(e), pursuant to the provisions of Public Law 233, approved October 8, 1951.

JANUARY-FEBRUARY, 1964 **Volume 29, Number 1**

An Official Publication of the Institute of Food Technologists

Journal of FOOD SCIENCE

JANUARY-FEBRUARY, 1964 Volume 29, Number 1

CONTENTS

Oleoresin of Capsicum (Red Chilies)—Some Technological and Chemical Aspects

G. L. TANDON, S. V. DRAVID, AND G. S. SIDDAPPA
Central Food Technological Research Institute, Mysore-2

(Manuscript received September 21, 1962)

SUMMARY

The principal source of the dry chili of commerce in India is *Capsicum annuum*, variety *acuminatum*. The oleoresin capsaicin, which is the alcoholic fraction of the ether extract of chilies, is the active ingredient of several essences and medicinal preparations from chilies. In the local variety used, the pericarp, which constitutes nearly 40% of the whole chilies, contains nearly 89% of the capsaicin, while the seeds contain 11%, and the stems only traces of it. Ether is one of the best solvents for extraction of the oleoresin by the percolation method. Since the pericarp contains considerably less fatty oil than the seeds, use of the pericarp for extraction obviates the difficulty of separation of the fatty oil from the oleoresin. The fatty oils, obtained separately from whole chilies and from the seeds, which are bland in taste, have higher acid and iodine values, but lower saponification values, than mustard or groundnut oils. Physicochemical standards have been determined for the oleoresin, fatty oil from whole chilies and fatty oil from the seeds alone.

Chilies are grown extensively in India. When the chilies are fully mature and red in color, they are harvested and dried in the sun. In 1960, Andhra Pradesh produced 95,000 tons, valued at 155 million rupees, and Mysore State 24,745 tons, valued at 62 million rupees (Anon, 1961a,b). While considerable confusion still exists in regard to classification of several varieties of chilies which occur in different shapes, sizes, shades of red and orange, and degree of pungency the variety *acuminatum (Capsicum annuum)* is the principal source of the dry chili of commerce in India (C.S.I.R., 1950). The fruits of this variety are bright red, slender and thin-walled. Other varieties grown are var. *grossum,* which includes "Bell Pepper"; var. *longum,* grown mainly for unripe fruit, var. *fastigatum,* and Bombay *Capsicum.* Bombay capsicum is grown mainly for pharmaceutical purposes.

Dry chili is used extensively as a condiment and also in several medicinal preparations for the treatment of lumbago, neuralgia, rheumatic disorder, etc. In most of these preparations, oleoresin capsaicin B.P.C., the alcoholic fraction of the ether extract of *Capsicum,* is the active ingredient. The tincture and essence of *Capsicum* are used to increase pungency in chewing and smoking tobacco, ginger, ginger ale, ginger soda, rum, etc. (Liverseege, 1932).

EXPERIMENTAL

Samples of dried chilies were obtained from the local market and are fairly representative of chilies grown in this region. All the three components—pericarp, seed, and stem—were separated by hand. For proximate analysis, further shredding was done in a micropulverizer (30-mesh).

Proximate analysis. Representative samples collected from 50-kg/bulk lots were used for analysis, Moisture, total ether extract, volatile ether extract, non-volatile ether extract, total ash, water-soluble ash, acid-insoluble ash, crude fiber, and protein were estimated according to methods of the AOAC (1955).

Minerals and vitamins. Calcium was estimated according to the method of the AOAC (1955); ascorbic acid by Robinson and Stotz (1945); iron by the method described by Snell and Snell (1954); and phosphorus by the method of Fiske and Subba Row (1925). The method of the Association of Vitamin Chemists (AVC., 1951) was followed for the estimation of total carotene and β-carotene using a Beckman quartz Model DU spectrophotometer.

Physicochemical characteristics. Refractive index, specific gravity, acid value, and saponification value were determined by the AOCS method (1946). Iodine value was estimated by Wij's method (Williams, 1950), and peroxide value by

Wheeler's method (1932). A Lovibond Tintometer was used to estimate visual color in the oleoresin prepared from the ether extract.

Estimation of capsaicin. Capsaicin content was determined by the method of North (1949) using a Klett Summerson photoelectric colorimeter (Model 8003), using a 62 filter to measure the blue color developed with phosphotungstic-phosphomolybdic acid reagent.

Method of preparation of oleoresin of Capsicum. In the laboratory trials, the method employed for the preparation of oleoresin of Capsicum was the one suggested by the Council of the Pharmaceutical Society of Great Britain (Anon., 1949), and also the method followed by the industry. The oleoresin was also prepared from the pericarp of chilies. Ether, alcohol, hexane, acetone, and chloroform were used as solvents. In the conventional methods, where whole chilies are used, a fatty oil, bland in taste, separates. When the pericarp alone was used, there was no such separation.

RESULTS AND DISCUSSION

Table 1 gives data on the physical composition, capsaicin content, ascorbic acid, minerals, and carotenoid pigments in whole chilies and their component parts. Table 2 gives the proximate analysis of the whole chilies and the component parts.

In Table 1, it can be seen that chilies, as used in these studies, contain nearly 40% pericarp, 54% seeds, and 6% stems. The pericarp contains nearly 89% capsaicin and the seeds 11%, with only traces in the stems. According to Tice (1933) the largest proportion of the pungent principle is concentrated in the dissepiment and, while there may be some in the epidermis, the seeds are entirely lacking in it. In the samples examined, it was possible that as the chilies were dry a small proportion of the inside placenta might have contaminated the seeds, resulting in a capsaicin content of 11% of the total in the seeds. The pericarp is thus the most suitable raw material for the preparation of oleoresin of Capsicum.

It can be seen from Table 1, that about 95% of the total coloring matter is in the pericarp alone. By the percolation method of extraction, it has been observed that ether extracts 66.5% of the total coloring matter, acetone 67.7%, hexane 58.1%, chloroform 61.1%, and alcohol 19.7%. Alcohol is thus a poor solvent for extraction of the coloring matter.

Table 1. Physical composition, capsaicin content, ascorbic acid, minerals and carotenoid pigments of whole chilies and component parts.[a, b, c]

	% of whole chilies	Capsaicin		Coloring matter			Ascorbic acid (mg/100 g)	Iron (mg/100 g)	Phosphorus (mg/100 g)	Calcium (mg/100 g)
		Total (μg/g)	Distribution (%)	Total (μg/g) as β-carotene	β-carotene (μg/g)	Distribution (%)				
Whole chilies	100.00	177	100.00	1733.0	125.0	100.00	28.99	10.24	356.6	210.0
		(150–193)		(1421–1812)	(100–148)		(18.9–31.2)	(6.3–12.4)	(299.2–382.4)	(198.3–224.6)
Pericarp	39.88	394	89.20	3977.0	261.4	94.6	38.62	5.92	262.8	200.0
	(35.1–42.0)	(354–412)	(88.1–91)	(3142–4020)	(198–286)		(27.1–42.4)	(4.3–7.4)	(214.1–276.2)	(183.2–226.2)
Seeds	54.10	35	10.80	17.9	3.3	4.9	15.33	11.37	464.1	70.0
	(49.0–56.1)	(30–37)	(9.1–11.2)	(12.0–18.3)	(1.6–4.3)		(12.1–19.3)	(5.6–13.9)	(412.2–492.6)	(58.2–73.2)
Stems	6.02	Traces	Traces	208.8	16.6	0.50	9.13	39.93	102.11	1600.0
	(5.3–8.9)	.....	.....	(191–220)	(12.0–19.2)		(6.2–9.8)	(30.2–43.7)	(89.1–108.4)	(1453.2–1632.0)

[a] Averages based on the analysis of five samples.
[b] Values expressed on moisture-free basis.
[c] Values in brackets indicate the range of variation.

Table 2. Proximate analysis of red chilies (local variety).[a, b]

Particulars	Moisture (%)	Ether extract (%)			Ash (%)			Protein (N × 6.25) (%)	Crude fiber (%)	Carbohydrates (by difference) (%)
		Total	Volatile	Non-volatile (fatty oil)	Total	Water soluble	Acid insoluble			
Whole chilies										
Max.	9.10	24.51	0.46	24.16	6.46	5.86	1.85	13.00	43.50	33.83
Min.	8.82	19.47	0.06	19.41	6.13	5.60	0.35	12.52	28.05	12.53
Av.	8.94	22.07	0.36	21.71	6.27	5.69	1.05	12.82	31.85	26.99
Pericarp										
Max.	7.81	18.56	0.84	7.77	10.07	8.86	0.35	13.18	35.57	26.06
Min.	7.31	17.88	0.49	7.29	9.64	8.36	0.08	12.26	34.16	22.62
Av.	7.55	18.19	0.65	7.53	9.78	8.59	0.23	12.72	34.96	24.35
Seeds										
Max.	6.44	26.75	0.69	26.67	5.27	3.03	0.14	16.36	39.83	22.99
Min.	6.09	24.64	0.01	23.95	3.98	2.17	0.11	16.01	32.38	11.79
Av.	6.29	26.03	0.31	25.71	4.46	2.54	0.13	16.21	37.19	16.11
Stems										
Max.	9.23	5.15	0.49	4.90	14.57	8.87	1.17	10.87	27.41	49.38
Min.	8.97	3.32	0.25	3.07	14.44	8.82	0.90	10.23	22.63	42.00
Av.	0.11	4.08	0.33	3.75	14.50	8.85	1.02	10.54	25.02	45.86

[a] Averages based on the analysis of five samples.
[b] Values expressed on moisture-free basis.

When the oleoresin is prepared by conventional methods, about 67% of the coloring matter is carried in the fatty oil, which separates on treatment with alcohol. The coloring matter dissolved in petroleum ether (b.p. 60–80°C) has maximum absorbance at 450 mμ and 452 mμ and this may sometimes be useful in detecting any added coloring matter in powdered chilies. The color substances of the fruits of the genus Capsicum were known to belong chemically to the carotenoids and Shuster and Lockhart (1954) had shown that the coloring matter extracted with acetone from Capsicum exhibited a characteristic absorption at 462 mμ and these measurements correlated well with a visible method based upon comparison with a color standard. The total coloring matter expressed as β-carotene in these samples (1421 to 1812 μg/g; Table 1) was found to be within the limits (900 to 4060 μg/g) reported by Pohle and Gregory (1960) in some samples of Capsicum spices and oleoresin of paprika examined by them.

Table 2 shows that the pericarp contains, on an average, 7.53%, seeds 25.71%, and stems 3.75% of bland non-volatile ether extract. The seeds are thus quite rich in fatty oil. When the oleoresin is prepared by conventional methods from whole chilies, considerable quantities of oil are to be separated subsequently to get the pure oleoresin. If the pericarp alone, which contains very little oil, is used for preparation of the oleoresin, the question of separation of this bland oil would not arise. Further, the oleoresin can be obtained directly by ether extraction alone, without the necessity of treatment with alcohol. Since the seeds do not contain any appreciable amount of capsaicin, oleoresin of satisfactory quality can be obtained without the use of seeds.

Oleoresin of Capsicum. During the preparation of oleoresin by the percolation method it was observed that the yield of extract was 17.46% with alcohol, 16.14% with ether, 15.6% with acetone, 15.04% with hexane, and 16.36% with chloroform. These variations in yield are due to extraction by the different solvents of constituents other than the oleoresin. The extracts had the following physicochemical characteristics.

	Range
Refractive index at 25°C	1.47200–1.47650
Specific gravity at 25°C	0.9286–1.0860
Acid value	35.5–65.0
Saponification value	57.0–101.0
Iodine value	125.0–195.2
Peroxide value	Nil
Capsaicin (mg/100 g)	701 with ether, 605 with hexane, 587 with chloroform, 523 with alcohol 515 with acetone

From the above, it can be seen that ether is the best solvent for the extraction of capsaicin, i.e., for the preparation of oleoresin of Capsicum.

The oleoresin prepared from the ether extract had the following composition: refractive index at 25°C, 1.47630; specific gravity at 25°C, 0.9556; acid value, 36.82; saponification value, 167.2; iodine value, 190.0; peroxide value, nil; capsaicin, 2010 mg/100 g; and Lovibond units: red, 11.2, and yellow, 9.9.

Fatty oil. Oil was extracted from the seeds alone, by using hexane as solvent, for a comparative study of the characteristics of the fatty oil. The fatty oil obtained from the seeds had the following constants. Refractive index at 25°C, 1.4690; specific gravity at 25°C, 0.9241; acid value, 9.4; iodine value, 145.6; and saponification value, 185. The fatty oil obtained from whole chilies had: refractive index at 25°C, 1.4780; specific gravity at 25°C, 0.9212; acid value, 16.28; iodine value, 142.4; and saponification value, 180. When compared with mustard and groundnut oils (Hattiangadi, 1958), the acid value and iodine value of the fatty oils from whole chilies and the seeds were higher than those of the other two oils, indicating more unsaturation. The saponification value of oils from the seed as well as oil from whole chilies was, however, lower.

CONCLUSIONS

Particle size determines the extraction capacity of chilies, the yield of oleoresin increasing with increasing fineness of the material.

Ether is the best solvent for extraction by percolation method.

In the industry, whole chili powder is used for the manufacture of oleoresin of Capsicum. The fatty oil, which is recovered as a by-product and is rich in color, is a waste product at present. Further, separation of this oil from the extract to recover the oleoresin, is an elaborate process.

The pericarp, which constitutes nearly 40% of the chilies, is the most important constituent for preparation of the oleoresin since it contains nearly 90% of the capsaicin. Use of the pericarp alone for preparation of the oleoresin has the following advantages.

a) Less of the solvent will be required for extraction.

b) Loss of solvent through evaporation will be reduced even when open containers are used, since the quantity of solvent used will be comparatively less.

c) Since the pericarp contains only 7.7% fatty oil, the problem of separation of this oil from the extract to recover pure oleoresin will not be a difficult one. Even if some of it separates, being quite small, it will not unduly affect the color of the oleoresin, especially for culinary purposes.

The seeds can be profitably employed as a condiment or for extraction of a colorless oil that can be used for edible purposes or for making soap.

Further work is in progress to study the nature of the flavoring material in green as well as in dry chilies and to separate the coloring matter from the oil obtained from whole chilies and find a use for it, since at present it is a waste product.

ACKNOWLEDGMENT

The authors are grateful to Dr. V. Subrahmanyan, Director, and Dr. Girdhari Lal, Assistant Director (Retd.), for keen interest and encouragement in this work, and to Dr. A. Sreenivasan, Deputy Director, Central Food Technological Research Institute, Mysore, for helpful criticism and guidance in preparing this paper.

REFERENCES

Anon. 1949. "The British Pharmaceutical Codex." Pharmaceutical Press, 17, Bloomsbury Square, London, W.C. 1.

Anon. 1961a. Spices Bull. Vol. I No. 2. Spice Export Promotion Council, Madras-1, India.

Anon. 1961b. Spices Bull. Vol. No. 3. Spice Export Promotion Council, Madras-1, India.

AOAC. 1955. "Official Methods of Analysis." Assoc. Agr. Chemists, Washington, D.C.

AOCS. 1946. "Official and Tentative Methods." American Oil Chemists' Society, Chicago 1.

AVC. 1951. "Methods of Vitamin Assay." Association of Vitamin Chemists, Interscience Publishers, Inc., New York.

CSIR. 1950. "Wealth of India—A Dictionary of Indian Raw Materials and Industrial Products." Vol. II, C.S.I.R., Delhi.

Fiske, C. H., and Y. Subba Row. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**, 375.

Hattiangadi, G. S. 1958. The Vanaspati Industry. The Hindustan Vanaspati Manufacturers, Bombay.

Liverseege, J. F. 1932. Adulteration and analysis of foods and drugs. J. & A. Churchill, London.

North, H. 1949. Colorimetric determination of capsaicin in oleoresin of Capsicum. *Anal. Chem.* **21**, 934.

Pohle, W. D., and R. L. Gregory. 1960. Color of Capsicum spices. *Food Tech.* **14**, 245.

Robinson, W. B., and E. Stotz. 1945. The indophenol-xylene extraction method for ascorbic acid and modifications for interfering substances. *J. Biol. Chem.* **160**, 217.

Shuster, H. V., and E. E. Lockhart. 1954. Development and application of objective methods for quality evaluation of spices. Color grading of spices. *Food Research.* **19**, 472.

Snell, F. D., C. T. Snell. 1954. "Colorimetric Methods of Analysis." 3rd ed. D. Van Nostrand Co., New York.

Tice, L. V. 1933. A simplified and more efficient method for the extraction of capsaicin together with the colorimetric method for the determination in Capsicum fruit and oleoresin. *Am. J. Pharm.* **105**, 320. *Cf.* Newman, A. A. 1953. Natural and synthetic pepper-flavored substances (3). Chemistry of capsaicin, the pungent principle of the Capsicum peppers, 16,413.

Wheeler, D. H. 1932. Peroxide formation as a measure of auto-oxidative deterioration. *Oil & Soap* **9**, 89.

Williams, K. A. 1950. "Oils, Fats and Fatty Foods; Wij's Iodine Value Method." 3rd ed. p. 108. J. & A. Churchill Ltd., London.

Carbonyls in Oxidizing Fat. VI. The Girard T Reagent in the Isolation and Determination of Micro Amounts of N-Aliphatic Aldehydes and 2-Alkanones

A. M. GADDIS, REX ELLIS AND G. T. CURRIE
Meat Laboratory
Eastern Utilization Research and Development Division
Agricultural Research Service
United States Department of Agriculture
Beltsville, Maryland

(Manuscript received April 27, 1963)

SUMMARY

Complete reaction of the Girard T reagent with n-alkanals, alk-2-enals, alk-2,4-dienals, and 2-alkanones of carbon chain length up to C-13 was obtained in anhydrous tertiary butyl alcohol. However, in practical applications in the presence of fat these reactions were greatly retarded and inhibited. Modification by the use of water in the reaction gave quantitative recovery of aldehyde homologous series from the fat. Quantitative or even uniform recovery of 2-alkanones could not be obtained. Optimum reaction with the Girard T reagent was at room temperature, and refluxing tended to decrease yields. Girard T hydrazones were converted quantitatively to 2,4-dinitrophenylhydrazones. The method is useful for the determination of aldehydes generated in autoxidized fats.

The Girard T reagent as a means of isolating carbonyl compounds was first reported by Girard and Sandulesco (1936). Valuable because of the water solubility of its hydrazones, this reagent has been used quite extensively for the isolation of carbonyl compounds from natural products (Wheeler, 1962). There have been a good many misconceptions regarding the Girard T reaction. Because of the reported relative ease of hydrolysis it was considered applicable only for ketones (Girard and Sandulesco, 1936) and therefore specific for that class of compounds. However, it has been shown to be effective for aldehydes, the derivatives being readily decomposed by mineral acid (Forss and Dunstone, 1954).

There is little in the literature on the completeness of reaction and subsequent recovery of aliphatic aldehydes and ketones. The mild conditions possible for the Girard T reagent are attractive for studies of the chemistry of oxidative fat rancidity, and of the related isolation and determination of free carbonyl compounds. Earlier work by the authors (Gaddis *et al.*, 1960) showed the possibilities of such use, but results did not check out in some instances, and a thorough study of the reagent's application was indicated. Nothing was known concerning the completeness of reaction with the various classes of carbonyl compounds involved, or of the efficiency of conversion of Girard T hydrazones to 2,4-dinitrophenylhydrazones. The latter derivatives have been shown to be useful for the characterization and measurement of n-aldehydes and 2-alkanones by already developed procedures (Ellis *et al.*, 1958; Ellis and Gaddis, 1959; Gaddis *et al.*, 1960; Gaddis and Ellis, 1959a,b).

Preliminary work has indicated that a major error may result from choice of reaction solvent in the Girard T reaction. Methanol and ethanol have been the standard solvents used since it was first reported. Braude and Forbes (1951) reported that 2,4-dinitrophenylhydrazine dehydrogenates primary and secondary alcohols containing two or more ethylenic or phenyl substituents in conjugation with the alcohol group, but observed no influence on saturated alcohols. Gaddis *et al.* (1961) found that both Girard T reagent and 2,4-dinitrophenylhydrazine tend to dehydrogenate or oxidize primary and secondary saturated alcohols in

micro amounts to their corresponding aldehydes or ketones. Tertiary butyl alcohol was found to be a relatively inert solvent that gave negligible blanks. This alcohol is also an excellent solvent for fats. Studies are described in this paper on the completeness of reaction of the Girard T reagent with n-alkanals, alk-2-enals, alk-2,4-dienals, and 2-alkanones under various conditions, and the capability of the reagent in the recovery of these types of compounds from fat.

EXPERIMENTAL

Solvents and reagents. Carbon tetrachloride, benzene, tertiary butyl alcohol, ethanol, methanol, and isopropanol were rendered carbonyl-free by treatment with 3 g trichloroacetic acid and 4–5 g 2,4-dinitrophenylhydrazine per liter, refluxing one hour, and distillation. Hexane was purified by the rapid method of Hornstein and Crowe (1962). The Girard T reagent was recrystallized twice from anhydrous purified ethanol, washed with tertiary butyl alcohol, and stored in a desiccator over concentrated sulfuric acid. The 2,4-dinitrophenylhydrazine reagent in *2N* HCl was made up fresh and purified as described by Gaddis *et al.* (1959). Authentic carbonyl compounds were distilled twice just before use, and their purity was checked by direct reaction with 2,4-dinitrophenylhydrazine reagent and paper chromatographic examination of the reaction mixture products (Gaddis and Ellis, 1959a). Alkanals and 2-alkanones were obtained from commercial sources. Alk-2-enals were prepared by the method of Radlove (1959), and alk-2,4-dienals by the procedure of Pippen and Nonaka (1958).

Methods. *General procedure.* The method used was to react 0.5–1.0 g of Girard T reagent in 20.0 ml of tertiary butyl alcohol (or other alcohol) containing an aliquot of a stock solution of the carbonyl compound (0.05–3 mg) in the alcohol.

To determine recovery from fat, 10.0 g of mildly rendered fresh lard were combined with the reaction mixture. To perform the reaction under aqueous conditions, 8.0 ml of water or buffer solution were added to the reaction mixture. For acid catalysis, 2.0 ml of glacial acetic acid (Girard and Sandulesco, 1936), 0.2 g Dowex 50 (Gaddis *et al.*, 1960), or Amberlite IRC 50 (Teitelbaum, 1958) was included. (Trade names are mentioned for identification, implying no endorsement.)

The reaction was conducted under an atmosphere of nitrogen by shaking at room temperature for a minimum of 3 hr. At the end of the reaction period, 150 ml of ice water (containing 0.9 equivalent of NaOH to acetic acid used) were added. After stirring, the contents of the reaction flask were transferred to a blender and agitated 1 min. The mixture was then transferred to a separatory funnel. The reaction flask and blender were washed with 50 ml of ice water, and this was added to the main portion. The contents of the separatory funnel were extracted three times with 35 ml of purified hexane. Reaction flask and blender were washed with 50 ml of hexane, which was added to the hexane extract. The hexane extract was washed with 50 ml of water.

The two aqueous portions were combined and added to 100 ml of 2,4-dinitrophenylhydrazine reagent. One hundred and twenty milliliters of *2N* HCl and 50 ml of carbon tetrachloride (optional in the case of aldehydes) were added, and the mixture was shaken well for 3 hr at room temperature.

Direct reaction of known amounts of monocarbonyl compounds with 2,4-dinitrophenylhydrazine was performed in two ways: 1) a 1.00-ml portion of a stock solution of the carbonyl compound in tertiary butyl alcohol was reacted with 25.0 ml of 2,4-dinitrophenylhydrazine reagent; 2) 20 ml of tertiary butyl alcohol containing the carbonyl compound were reacted with 100 ml of 2,4-dinitrophenylhydrazine reagent, 120 ml of *2N* HCl, and 250 ml of water, the latter conditions being those used for the Girard T hydrazone conversion and necessary to compare with reaction of the Girard T reagent. The method of reaction in diluted *2N* HCl is a modified Iddles *et al.* (1939) procedure and is similar to that described in earlier publications.

The 2,4-dinitrophenylhydrazone derivatives were extracted with carbon tetrachloride and benzene (Gaddis *et al.*, 1959), and monocarbonyl derivatives were purified by passage through hydrated alumina as described in previous publications (Gaddis *et al.*, 1959, 1960; Gaddis and Ellis, 1959).

When determinations were made on fat, the grade of alumina and solvent systems described by Schwartz and Parks (1961) and Keeney *et al.* (1962) were used to obtain clean separation of the simple monocarbonyl derivatives from the ketoglyceride derivatives. Appropriate blanks were run for all steps and checked for artifacts.

The amount of aldehyde or ketone was determined by obtaining absorption data and calculating micromolar amounts from average extinction coefficients. All absorbance values were based on the amount in 100.0 ml of carbon tetrachloride solution, and absorbance values used for 1 micromole were 0.205/343–52 mμ for 2-alkanones and alkanals, 0.276/355–65 mμ for alk-2-enals, and 0.373/370–380 for alk-2,4-dienals (Ellis and Gaddis, 1959).

RESULTS AND DISCUSSION

Reaction of Girard T reagent with carbonyl compounds. The completeness of reaction of the Girard T reagent with carbonyl compounds was evaluated by conversion of the hydrazone derivatives to 2,4-dinitrophenylhydrazones. Condensation of n-aliphatic aldehydes and 2-alkanones with 2,4-dinitrophenylhydrazine is generally considered to be quantitative. However, Begemann and De Jong (1959) studied methods of reaction reported in the literature for small amounts of aldehydes and ketones and found much variation in completeness of reaction. Those investigators showed that passing a solution of carbonyl compound in petroleum ether through a Celite column charged with 2*N* HCl and 2,4-dinitrophenylhydrazine gave quantitative results consistently. Direct reaction of aldehydes and 2-alkanones with 2,4-dinitrophenylhydrazine by procedure 1 gave quantitative results. This method was used to check the contents of monocarbonyl compounds in stock solutions. Table 1 shows the completeness of condensation of n-aliphatic aldehydes with 2,4-dinitrophenylhydrazine by procedure 2, the method employed in this work. These data indicate that reaction under these conditions was virtually quantitative. However, as shown in Table 2, the formation of 2-alkanone derivatives by similar reaction conditions was very much less satisfactory. With the exception of acetone, 2-alkanones with carbon chains up to about C-8 reacted poorly. The great dilution necessary in the Girard T procedure was apparently responsible for this (Cheronis *et al.*, 1957). In connection with this result, Stone *et al.* (1962) reported that 2-heptanone by the gravimetric method of Iddles *et al.* (1939) reacted only to the extent of 63%. Addition of 50 ml of carbon tetrachloride to the reaction mixture brought all yields to a quantitative level. The reason for the effectiveness of the carbon tetrachloride extraction during the reaction may be the breaking of an equilibrium by removal of hydrazones as formed. The higher-molecular-weight 2-alkanones form hydrazones that are more insoluble in the reaction mixture, and the condensation goes to completion. The solvent extraction did not affect recoveries of n-aldehydes and the higher alkanones, and was used regularly in the remaining experiments.

Table 1. Reaction of aldehydes with 2,4-dinitrophenylhydrazine.

n-Aldehydes	Weight (mg)	Absorbance		
		Theoretical	Found	% Recovery
Alkanals				
C-4	1.088	3.075	3.145	102
	0.242	0.688	0.658	96
C-5	0.242	0.576	0.547	95
C-7	1.758	3.16	3.19	101
	0.244	0.439	0.410	93
	0.049	0.088	0.080	91
C-9	0.248	0.357	0.322	93
C-10	0.468	0.614	0.618	101
C-12	0.25	0.278	0.252	91
	0.05	0.056	0.061	109
Alk-2-enal				
C-4	0.571	2.25	2.268	101
	0.258	1.014	0.997	98
C-7	0.963	2.37	2.32	98
C-10	1.094	1.96	1.84	94
Alk-2,4-dienal				
C-6	0.596	2.314	2.372	106
C-9	0.282	0.76	0.768	101
C-10	1.040	2.55	2.43	95
C-15	1.305	2.195	2.06	94

The reaction of n-aliphatic aldehydes with the Girard T reagent at room temperature under anhydrous conditions is shown in Table 3. The data indicate virtually quantitative reaction under neutral conditions or in the presence of acetic acid, except for very high-molecular-weight aldehydes. The amount of carbonyl compound present was determined by direct reaction with 2,4-dinitrophenylhydrazine; therefore, it follows that the conversion of Girard T hydrazone to 2,4-dinitrophenylhydrazone was very effective. The reaction appeared not to be influenced by the presence of acetic acid. Acetic acid was originally recommended for the Girard T reaction (Girard and Sandulesco, 1963) and definitely aids the condensation with some 2-alkanones.

The completeness of reaction of 2-alkanones at room temperature under neutral and anhydrous conditions was related to the molecular weight. As shown in Table 4, acetone reacted quantitatively; but, with increase in carbon chain, there was a steady decline in completeness of combination. The

Table 2. Reaction of 2-alkanones with 2,4-dinitrophenylhydrazine.

2-Alkanone	Weight (mg)	Absorbance				
		Theoretical	Found	%	Carbon tetrachloride[b] Found	%
C-3	1.022	3.612	3.400	95	3.72	103
	0.235	0.830	0.788	94	....	....
C-4	1.110	3.163	1.92	61	3.40	107
C-5	1.412	3.366	1.58	47	3.42	102
	0.241	0.573	....	....	0.622	109
C-7	1.874	3.370	2.15	64	3.32	99
	0.285	0.515	....	....	0.546	106
C-9	2.36	3.407	3.470	102	....	....
C-11	2.62	3.161	3.370	107	....	....
C-13	0.248	0.250	0.269	106	....	....
	3.390	3.506	3.650	104	....	....
C-17	3.950	3.188	3.220	101	....	....
C-18	4.395	3.19	3.25	102	....	....

[a] Carbon tetrachloride in reaction mixture.

prescribed use of acetic acid increased yields to a quantitative level up to about C-11, but showed small benefit for the higher-molecular-weight ketones. This appears not to be due to an equilibrium state, since the high-molecular-weight aldehydes also reacted incompletely.

Recovery of carbonyl compounds from

Table 3. Reaction of n-aldehydes with Girard T reagent under anhydrous conditions.

Aldehydes	Absorbance				
	Present	Neutral Found	Neutral % recovered	Acetic acid Found	Acetic acid % recovered
n-Alkanals	0.118	0.114	97	....	....
C-3	0.123	0.115	94	0.132	107
	0.643	0.621	97	0.641	100
	0.780	0.800	103	....	....
C-4	0.622	0.636	102	0.621	100
C-5	0.547	0.534	98	0.606	111
	2.040	1.970	97	1.870	91
C-7	0.084	0.080	95	....	....
	0.388	0.374	96	0.418	108
	2.570	2.500	99	....	....
C-9	0.308	0.319	104	0.349	113
C-10	2.320	2.244	97	2.322	100
C-12	0.058	0.049	85	....	....
	0.262	0.266	102	0.283	108
C-16	2.45	1.010	41	1.335	54
Alk-2-enal					
C-4	0.196	0.203	104	....	....
	0.997	1.022	103	1.03	103
C-9	5.442	5.03	92	....	....
C-10	2.212	2.01	91	2.215	100
	2.212	2.09	95	....	....
Alk-2,4-dienal:					
C-9	0.768	0.730	95	0.750	100
C-10	2.015	1.987	99	....	....
	2.158	....	....	1.87	87
C-15	2.158	1.405	65	1.055	49

Table 4. Reaction of 2-alkanones with Girard T reagent under anhydrous conditions.

	Absorbance				
		Neutral		Acetic acid	
2-Alkanones	Present	Found	% recovered	Found	% recovered
C-3	0.778	0.779	100	0.770	99
	3.580	3.690	103	.....	.....
C-5	0.622	0.515	83	0.610	98
	3.334	3.215	97	3.360	101
C-7	0.546	0.352	65	0.518	95
	3.338	2.865	89	3.190	96
C-9	3.407	2.170	63	3.200	94
C-11	3.161	1.460	46	3.020	96
C-13	2.548	1.14	45	1.455	57
C-17	2.318	1.070	46	1.555	67

fat. The next step in the investigation was to determine the effectiveness of the Girard T reagent in recovery of these types of carbonyl compounds from fat. The difficulties encountered in this practical application were quite unexpected. As indicated in Table 5, the reaction of aldehydes under neutral and anhydrous conditions was retarded to an extent related to chain length and degree of unsaturation. Aldehydes of C-1 to C-12 chain length showed quantitative to poor recovery, and the high-molecular-weight compounds, as usual, reacted poorly. The use of acetic acid made yields of C-1 to C-12 aldehydes fairly uniform at a near quantitative level. The reaction of most of the 2-alkanones was greatly inhibited by the fat. Acetic acid improved yields, but did not make recovery uniform for the ketones in the volatile range. It would not be possible, then, to obtain a representative recovery of a mixture of methyl ketones. The use of acetic acid is advantageous in this respect with aldehydes, but for some applications its use might be objectionable.

Other investigators (quoted by Lea and Swoboda, 1962) have observed a declining tendency of carbonyl compounds with increase in molecular weight to react with certain carbonyl reagents. Stenlake and Williams (1957) used the Girard T reagent in ethanol and had difficulty in quantitatively removing carbonyl compounds from lemon oil. They considered this to be due to a solvent effect that was rectified by changing the proportions of the reaction components. At best, tertiary butyl alcohol is a barely adequate solvent for the Girard T reagent, and solution of the fat appeared to throw most of the dissolved reagent out. A more polar reaction medium might be required. Methanol or ethanol is objectionable because of already stated reasons (Gaddis *et al.*, 1961), and reports indicate similar objections in the case of glycerol and glycols (Welti and Whittaker, 1962). Isopropyl alcohol used recently by Stanley *et al.* (1961) would also be employed with reluctance. Water remains, but the literature has generally implied that the reaction should be conducted under anhydrous conditions. However, Wheeler *et al.* (1961) reported the successful use of water in kinetic studies of the Girard T reaction.

Effect of water in the reaction mixture. The very favorable effect of water in the reaction mixture on recovery of aldehydes from fat is shown in Table 6. There was considerable variation in the requirements of individual members of the different aldehyde classes for volume of water and reaction time. Over-all, 8.0 ml of water and 3 hr of reaction time were found to give quantitative results. For the first time, reaction was nearly complete with the high-molecular-weight aldehydes. Acetic acid under aqueous conditions depressed the yield of the lower-molecular-weight aldehydes to a variable degree, and greatly lowered the recovery of high-molecular-weight compounds. This was probably due to a reversible effect of low pH. Tests with citric acid-disodium phosphates showed no reaction reversal at

Table 5. Recovery from fat under anhydrous conditions with Girard T reagent.

Monocarbonyl compounds	Absorbance				
		Neutral		Acetic acid	
	Present	Found	% recovered	Found	% recovered
Alkanal					
C-3	1.741	1.639	94	.....	.....
C-5	1.391	1.382	99	.....	.....
	1.786	1.724	97	.....	.....
	2.600	.....	.....	2.315	89
	2.170	.....	.....	1.995	92
C-7	1.861	1.699	91	.....	.....
	1.978	1.814	92	.....	.....
C-10	3.054	2.034	67	.....	.....
C-12	2.090	.....	.....	2.035	97
	2.158	1.53	71	.....	.....
C-16	2.450	0.74	30	1.615	66
Alk-2-enal					
C-4	3.950	3.244	82	.....	.....
	2.272	.....	.....	1.970	87
	2.272	.....	.....	1.845	81
C-7	2.303	1.008	44	.....	.....
	2.372	.....	.....	2.14	90
C-10	2.212	.....	.....	2.34	106
	1.878	1.000	53	.....	.....
Alk-2,4-dienal					
C-6	2.328	1.928	83	.....	.....
	2.180	.....	.....	2.00	92
C-10	2.403	0.776	32	.....	.....
	2.140	.....	.....	1.96	92
	2.238	.....	.....	1.96	88
C-15	2.158	0.22	10	0.82	38
2-Alkanone					
C-4	2.061	1.990	97	.....	.....
C-7	2.138	0.350	16	1.575	74
	2.138	0.240	11	1.465	69
C-11	2.658	0.170	6	1.015	38
C-13	2.548	0.035	1	0.541	21
C-17	2.318	0.00	0	0.145	6

pH 3.0, but a slowing of reaction rate took place above pH 5.0. This was not investigated further, since satisfactory reaction of aldehydes was obtained under unbuffered conditions.

Reaction of 2-alkanones with Girard T under aqueous conditions in the presence of fat was poor. As shown in Table 7, yields decreased with length of carbon chain, and acetic acid again depressed the degree of reaction. Tests with pH 3.0–7.0 buffers and 0.1–0.2 mole sodium acetate with acetic acid showed some small improvement in recovery at the higher pH range. Reaction had reached equilibrium in less than 2 hr, and extension of reaction times to as long as 16 hr did not improve yields significantly.

Recovery of 2-alkanones. Achievement of quantitative or even uniform recovery of 2-alkanones from fat is elusive. However, these compounds from C-3 to C-11 reacted quantitatively with Girard T reagent in the presence of acetic acid under anhydrous conditions without the fat (Table 4). Investigators (Wheeler, 1962) have used ethanol or methanol for other applications with a degree of success. The effect of solvents when the reaction was run in the presence of fat under anhydrous conditions is shown in Table 8. Ethanol and methanol improved

Table 6. Recovery of aldehydes from fat under aqueous conditions with Girard T reagent.

	Absorbance				
		Neutral		Acetic acid	
n-Aldehydes	Present	Found	% recovered	Found	% recovered
Alkanal					
C-3	1.741	1.756	101	....	....
C-5	1.391	1.391	100	....	....
	2.600	....	....	2.230	86
C-7	1.861	1.843	99	....	....
C-10	3.054	2.968	97	....	....
C-12	2.09	....	....	1.65	79
C-16	1.90	1.62	85	....	....
	2.45	2.11	86	0.72	29
Alk-2-enal					
C-4	2.072	1.995	96	....	....
	2.272	....	....	0.83	37
C-7	1.772	1.725	97	....	....
	2.372	....	....	2.010	85
C-10	1.842	1.790	97	....	....
	2.122	....	....	1.730	78
Alk-2,4-dienal					
C-6	2.31	2.17	95	....	....
	2.18	....	....	2.165	99
C-10	2.06	2.07	101	....	....
	2.238	....	....	2.084	93
C-15	2.060	1.92	93	....	....
	2.158	....	....	0.605	28

reaction considerably, though not to a quantitative level. Ethanol or methanol might be useful in obtaining a more representative isolation of lower-molecular-weight 2-alkanones. Acetic acid or a longer reaction time of 16 hr did not increase the yield very appreciably except in the case of tertiary butyl alcohol and isopropyl alcohol. Isopropyl alcohol, used recently by Stanley *et al.* (1961), favored 2-alkanone isolation somewhat better than tertiary butyl alcohol.

Effects of solvent, temperature and acid. A comparison of the use of the four different solvents for fat under anhydrous conditions shown in Table 9 indicates a lack of uniformity in reaction with representatives of the three aldehyde classes. The primary alcohols accelerated reaction but were incomplete with alkanal C-12 after 16 hr of reaction. Tertiary butyl alcohol determinations were incomplete with alkanal C-12 and alk-2-enal C-10, but quantitative with alk-2,4-dienal C-10 at the longer reaction time.

Girard and Sandulesco (1936) originally recommended refluxing, and this step has

Table 7. Reaction of 2-alkanones with Girard T under aqueous conditions in the presence of fat.

	Absorbance				
		Neutral		Acetic acid	
2-Alkanones	Present	Found	% recovered	Found	% recovered
C-4	2.061	2.105	102	....	....
	2.228	....	....	1.86	84
C-7	2.138	1.100	51	0.99	46
C-11	2.658	1.27	48	0.67	25
C-13	2.548	0.44	17	0.35	14
C-17	2.318	0.30	13	0.04	2

Table 8. The effect of anhydrous solvent on reaction of C-11 2-alkanones in the presence of fat with Girard T reagent.

	Absorbance					
		Neutral			Acetic acid	
Solvent (20 ml)	Present	Found	% recovered		Found	% recovered
3-hr reaction						
Tertiary BuOH	2.658	0.13	5	2.658	0.575	22
Isopropyl alcohol	2.658	0.20	8	2.578	0.81	32
Ethyl alcohol	2.578	1.47	57	2.578	1.75	69
Methyl alcohol	2.678	1.18	44	2.678	1.435	54
16-hr reaction						
Tertiary BuOH	2.658	0.145	6			
Isopropyl alcohol	2.578	0.746	29			
Ethyl alcohol	2.578	1.37	53			
Methyl alcohol	2.678	1.37	51			

been more or less adhered to through the intervening years. This work indicated that, for the compounds examined, refluxing was either unnecessary, ineffective, or detrimental. Heating actually tended to reduce recovery, and the effect was particularly sharp in the case of alk-2-enals and alk-2,4-dienals. An adverse affect of heat in the Girard T reaction has also been commented on by Wheeler *et al.* (1961). Where reaction was reluctant, as in the 2-alkanones, there was no indication that refluxing aided reaction. Where primary and secondary alcohols were used for solvents, refluxing greatly increased the amount of dehydrogenation (Gaddis *et al.*, 1961) and the carbonyl compound artifacts. For some applications, it is fortunate that the reaction goes without heat, which would break down carbonyl precursors (Gaddis *et al.*, 1960).

Teitelbaum (1958) suggested using a cation-exchange resin, Amberlite IRC 50, as a substitute for glacial acetic acid. This modification has been used recently by several investigators (Gaddis *et al.*, 1960, Stanley *et al.*, 1961). Amberlite IRC 50 and a more acid cation-exchange resin, Dowex 50, were used in numerous comparisons under the conditions described above. They showed nothing of advantage as cata-

Table 9. The effect of anhydrous solvent on the reaction of aldehydes in the presence of fat with Girard T reagent.

	Absorbance				
		Reaction 3 hr		Reaction 16 hr	
Solvent (20 ml)	Present	Found	% Recovery	Found	% Recovery
Alkanal C-12					
Tertiary BuOH	2.158	1.53	71	1.32	61
Isopropyl alc.	2.228	0.60	27	1.135	51
Ethanol	2.228	1.71	77	1.65	74
Methanol	2.198	1.84	84	1.86	85
Enal C-10					
Tertiary BuOH	1.878	1.00	53	1.27	68
Isopropyl alc.	1.618	1.40	86	1.52	94
Ethanol	1.908	1.88	99	2.11	109
Methanol	1.918	1.59	82	1.54	79
2,4-Dienal C-10					
Tertiary BuOH	2.158	1.01	47	2.105	98
Isopropyl alc.	1.178	1.58	73	1.925	88
Ethanol	2.228	1.95	88	2.200	99
Methanol	2.228	1.59	71	2.12	95

lysts for the condensation. Amberlite IRC 50 was completely ineffective as a replacement for glacial acetic acid in the reaction of 2-alkanones with Girard T reagent. These resins, especially the Dowex 50, tended to decrease recoveries, and this was particularly serious in the case of the unsaturated aldehydes. Some of this loss may be due to irreversible holding of the Girard T hydrazone by the resin (Hornstein, 1962). On the other hand, the Dowex 50 resin has shown promise as a useful means of decomposition of hydroperoxides into carbonyl compounds (Gaddis *et al.*, 1960).

Sensitivity and utility of the method. The sensitivity of the determination began to fall off at concentrations lower than 0.5 μm carbonyl/100 ml. This decline was due to the increasing significance of variation in the blank. The blank was composed of unremovable impurities formed from the carbonyl reagents. These reaction by-products had an absorption maximum in carbon tetrachloride at about 340 mμ.

The method is believed to be useful for the isolation of aldehydes from oxidized fats and oils. Complete evaluation of the reagent's effect on hydroperoxides and other oxidative carbonyl precursors is considered beyond the scope of this paper. It has been established that aldehydes can be isolated quantitatively from fat by this procedure. In view of earlier work by the authors (Gaddis *et al.*, 1960), the mild conditions employed might be expected to recover aldehydes to a degree approaching those that are free. Removal of only the free carbonyl compounds may be an elusive achievement. According to Lea and Swoboda (1962), there appears to be some formation or release of volatile carbonyl compounds from non-volatile precursors even under the mild conditions of vacuum distillation at 50°C. This may explain the isolation of more carbonyl compounds upon repeated treatment with the Girard T reagent in our earlier study (Gaddis *et al.*, 1960). Preliminary studies have shown that this Girard T reagent procedure extracts much less aldehyde than the steam distillation method, and that values approach those obtained by the vacuum distillation technique of Lea and Swoboda (1962). The Girard T reagent under these mild optimum conditions hydrolyzes aldehyde diethyl acetals. However, it does not split the enol ether-linked aldehydes present in fresh fats (Schogt *et al.*, 1960; Parks *et al.*, 1961). Reaction with the naturally occurring ketoglycerides (Keeney *et al.*, 1962) was incomplete.

REFERENCES

Begemann, H. P., and K. De Jong. 1959. Analytical applications of a celite/dinitrophenylhydrazine column. *Rec. trav. chim.* **78**, 275.

Braude, E. A., and W. F. Forbes. 1951. Oxidation of alcohols by 2,4-dinitrophenylhydrazone. *J. Chem. Soc.* **1951**, 1762.

Cheronis, N. D., H. Stein, and V. M. Levey. 1957. Derivatives of small quantities of organic compounds and lower limits of organic reaction. *Microchem. J.* **1**, 39.

Ellis, R., and A. M. Gaddis. 1959. Paper chromatography of 2,4-dinitrophenylhydrazones. Estimation of 2-alkanone, n-alkanal, alk-2-enal and alk-2,4-dienal derivatives. *Anal. Chem.* **31**, 1997.

Ellis, R., A. M. Gaddis, and G. T. Currie. 1958. Paper chromatography of 2,4-dinitrophenylhydrazones of saturated aliphatic aldehydes. *Anal. Chem.* **30**, 475.

Forss, D. A., and E. A. Dunstone. 1954. The Girard reaction. *Nature* **173**, 401.

Gaddis, A. M., and R. Ellis. 1959a. Paper chromatography of 2,4-dinitrophenylhydrazones. Resolution of 2-alkanone, n-alkanal, alk-2-enal and alk-2,4-dienal derivatives. *Anal. Chem.* **31**, 870.

Gaddis, A. M., and R. Ellis. 1959b. Carbonyls in oxidizing fat. II. The identity and amounts of steam volatile monocarbonyls in a rancid freezer-stored pork fat. *Food Research* **24**, 392.

Gaddis, A. M., R. Ellis, and G. T. Currie. 1959. Carbonyls in oxidizing fat. I. Separation of steam volatile monocarbonyls into classes. *Food Research* **24**, 283.

Gaddis, A. M., R. Ellis, and G. T. Currie. 1960. Carbonyls in oxidizing fat. III. The distribution of volatile and non-volatile carbonyls. *Food Research* **25**, 495.

Gaddis, A. M., R. Ellis, and G. T. Currie. 1961. The Girard T reagent for carbonyls. *Nature* **191**, 1391.

Girard, A., and G. Sandulesco. 1936. Sur une nouvelle série de réactifs du groupe carbonyle, leur utilisation à l'extraction des substances cétoniques et à la caractérisation microchemique des aldéhydes et cétones. *Helv. chim. Acta* **19**, 1095.

Hornstein, I. 1962. Private communication.

Hornstein, I., and P. F. Crowe. 1962. Carbonyl removal from nonoxygenated solvents. *Anal. Chem.* **34**, 1037.

Iddles, H. A., A. W. Low, B. D. Rosen, and R. T. Hart. 1939. Determination of carbonyl compounds by means of 2,4-dinitrophenylhydrazine. Water-insoluble carbonyl compounds. *Ind. Eng. Chem., Anal. Ed.* **11**, 102.

Keeney, M., I. Katz, and D. P. Schwartz. 1962. Occurrence of ketostearic acids in cow's milk fat: suggested intermediates in unsaturated fatty acid biosynthesis. *Biochim. et Biophys. Acta* **62**, 615.

Lea, C. H., and P. A. T. Swoboda. 1962. Simple vacuum distillation procedure for determination of the volatile carbonyl content of autoxidizing edible fats. *J. Sci. Food Agr.* **13**, 148.

Parks, O. W., M. Keeney, and D. P. Schwartz. 1961. Bound aldehydes in butter oil. *J. Dairy Sci.* **44**, 1940.

Pippen, E. L., and M. Nanaka. 1958. A convenient method for synthesizing normal aliphatic 2,4-dienals. *J. Organic Chem.* **23**, 1580.

Radlove, S. B. 1958. The synthesis of 2-heptenal. *J. Amer. Oil Chemists' Soc.* **35**, 236.

Schogt, J. C. M., P. H. Begemann, and J. Koster. 1960. Nonphosphatide aldehydogenic lipids in milk fat, beef tallow, and ox heart. *J. Lipid Research* **1**, 446.

Schwartz, D. P., and O. W. Parks. 1961. Preparation of carbonyl-free solvents. *Anal. Chem.* **33**, 1396.

Stanley, W. L., R. M. Ikeda, S. H. Vannier, and L. A. Rolle. 1961. Determination of the relative concentrations of the major aldehydes in lemon, orange and grapefruit oils by gas chromatography. *J. Food Sci.* **26**, 43.

Stenlake, J. B., and W. D. Williams. 1957. The spectrophotometric determination of α,β-unsaturated aldehydes and ketones with Girard T reagent. I. Essential oils. *J. Pharm. and Pharmacol.* **9** 900.

Stone, H., C. S. Ough, and R. M. Pangborn. 1962. Determination of odor difference thresholds. *J. Food Sci.* **27**, 197.

Teitelbaum, C. L. 1958. Isolation of carbonyl compounds under neutral conditions using the Girard reagent. *J. Org. Chem.* **23**, 646.

Welti, D., and D. Whittaker. 1962. The oxidation of glycols with 2,4-dinitrophenylhydrazine hydrochloride. *Chem. & Ind.* **22**, 986.

Wheeler, O. H. 1962. The Girard reagents. *Chem. Rev.* **62**, 205.

Wheeler, O. H., V. S. Gaind, and O. Rosado. 1961. Formation of Girard T derivatives. *J. Org. Chem.* **26**, 3537.

Reactions Involved in Sulfite Bleaching of Anthocyanins

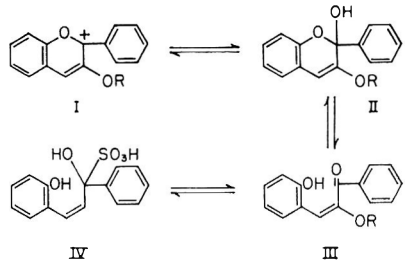
LEONARD JURD
Western Regional Research Laboratory,[a] Albany 10, California

(Manuscript received November 9, 1962)

SUMMARY

The bleaching of anthocyanins by sulfur dioxide is a reversible process that does not involve hydrolysis of the 3-glycosidic group, reduction of the pigment, or addition of bisulfite to a ketonic, chalcone derivative. In sulfite decoloration the reactive species is the anthocyanin carbonium ion (R^+). The experimental evidence indicates that this simply reacts with a bisulfite ion to form a colorless chromen-2 (or 4)-sulfonic acid (R-SO_3H), similar in structure and properties to an anthocyanin carbinol base (R-OH).

Although bleaching of anthocyanin pigments by sulfur dioxide is commonly encountered in commercial fruit processing, the reactions involved are obscure. In the bleaching of discolored foodstuffs it is generally assumed (Stadtman, 1948) that sulfur dioxide is effective by virtue of its reducing properties or by the formation of additive compounds with substances possessing active carbonyl groups. On the basis of limited experimental evidence, both of these theories have been applied to account for anthocyanin decoloration. Anthocyanins are readily reduced by a variety of reagents to colorless flavans (e.g., Freudenberg *et al.*, 1925; Charlesworth *et al.*, 1933; Elstow and Platt, 1950). However, Genevois (1956) and, more recently, Ribéreau-Gayon (1959) noted that sulfite decoloration of anthocyanins at pH 3 is rapidly reversible on acidification to pH 1 or on the addition of such carbonyl reagents as acetaldehyde. Those authors therefore pointed out that a reduction (Sannié and Sauvain, 1952) does not occur, and adopted the alternate view that an active carbonyl group was involved. Since the rate and extent of sulfite decoloration is pH-dependent, Ribéreau-Gayon suggested: a) that in aqueous solution at pH 3 an anthocyanin (I) is in equilibrium with its carbinol base (II) and the carbonyl derivative (chalcone) (III), and b) that the principal decoloration reaction was the formation of a chalcone-bisulfite additive compound IV, thus shifting the equilibrium to the right.



Spectral examination of the equilibrium reactions and sulfite decoloration of both natural and synthetic flavylium salts, however, has now shown unequivocally that chalcones are not involved in sulfite decoloration, the reactive species being the flavylium carbonium ion (I).

EXPERIMENTAL

Anthocyanins and flavylium salts. Cyanidin, pelargonidin 3-methyl ether, and the synthetic flavylium salts used in this investigation are known compounds. They were prepared in accordance with the general methods described by Robinson et al., viz., acid condensation of appropriate *o*-hydroxybenzaldehyde and acetophenone derivatives in ethyl acetate-ethanol (Robertson and Robinson, 1928) or glacial acetic acid solutions (Perkin *et al.*, 1908).

Specimens of pure natural anthocyanins—pelargonidin 3-glucoside and cyanidin 3-rhamnoglucoside—were respectively provided by Drs. E. Sondheimer and J. B. Harborne.

Determination of spectra. Stable, standard solutions of the anthocyanins and flavylium salts in 0.5% ethanolic HCl were prepared containing 0.120–0.130 g/L. One-ml portions of these solutions were diluted to 25.0 ml with aqueous buffers. For the determination of equilibrium reactions the buffered solutions were allowed to stand 2–3 hr at room temperature and their pH and spectra then measured on a Beckman DK2 recording spectrophotometer in 1-cm silica cells. In studying

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

sulfite decoloration, sodium bisulfite (20 or 200 ppm) was added to similarly buffered solutions as described in the text.

RESULTS AND DISCUSSION

In this laboratory, the experimental observations of Genevois and Ribéreau-Gayon on the sulfite decoloration of *natural* anthocyanins were first confirmed. For example, pure cyanidin 3-rhamnoglucoside V ($\lambda_{max.}$ 510, $\log_e$ 4.47 in aqueous solution at pH 0.7) was completely decolorized on the addition of sodium bisulfite (80 ppm) in an aqueous buffer, pH 3.04. Re-acidified to pH 0.7, the decolorized solution rapidly gave 95.6% recovery of pigment (measured after 1 hr). The recovered product was identical spectrally and chromatographically with cyanidin 3-rhamnoglucoside (R_f .64 (water, acetic acid, conc. HCl, 80:40:5 V/V, .74 (Forrestal solvent), .20 (*n*-BuOH/2 N HCl)). These properties differed distinctly from those of cyanidin, the aglycone of the glycoside ($\lambda_{max.}$ 514 mμ; R_f .29, .53, .69 in the above solvents). Thus, these data confirm that reduction does not occur, and, furthermore, eliminate the possibility that decoloration involves an initial hydrolysis of the 3-glycosidic grouping to give a potentially ketonic anthocyanidin aglycone.

Unlike unconjugated carbinol bases II, which show little absorption above 300 mμ, chalcones are yellow compounds that absorb intensely in the region of 340–400 mμ (Geissman, 1955; Jurd and Horowitz, 1961). The spectra of cyanidin 3-rhamnoglucoside (without added bisulfite) at equilibrium in aqueous solutions in the pH range 1–4, however, give no indication of chalcone formation. In Fig. 1, it is apparent that the intensity of absorption at 510 mμ, the $\lambda_{max.}$ of the salt, decreases markedly as the pH is raised *without* a simultaneous increase in intensity of absorption at 350–400 mμ. These spectral changes should be compared with those of flavylium salts, unsubstituted in the 3-position, discussed below. The curves in Fig. 1, therefore, indicate that the decrease in the anthocyanin salt V concentration at higher pH is due only to the formation of increasing quantities of a stable carbinol base, e.g., VI. Similar spectral changes were

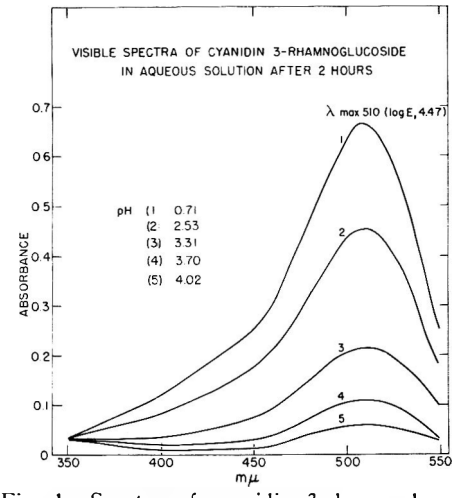
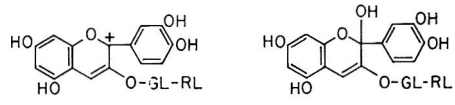


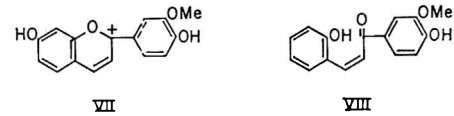
Fig. 1. Spectra of cyanidin 3-rhamnoglucoside (1.6×10^{-2} g/L) at equilibrium in buffered aqueous solutions.

observed with pelargonidin 3-glucoside and 3-methyl ether.



V VI

In contrast to natural anthocyanins, it has recently been demonstrated (Jurd, 1963) that a flavylium salt $\rightleftharpoons$ chalcone equilibrium definitely exists in aqueous solutions of flavylium salts unsubstituted in the 3 position. The presence of chalcones at equilibrium in these solutions is easily recognized by the formation of distinct absorption maxima in the region of 350–400 mμ. Representative spectra of 3′-methoxy-4′,7-dihydroxyflavylium chloride (VII), for example, at equilibrium in the pH range 1–5, are reproduced in Fig. 2. It is apparent that at higher pH's, as the intensity of absorption at 468 mμ (the $\lambda_{max.}$ of the salt) decreases, absorption at 373 mμ progressively increases in direct proportion to the quantity of the chalcone (VIII) being formed. This and other unsubstituted flavylium salts, therefore, provide an excellent means for determining simply and unambiguously the relative reactivities of the carbonium ion VII



VII VIII

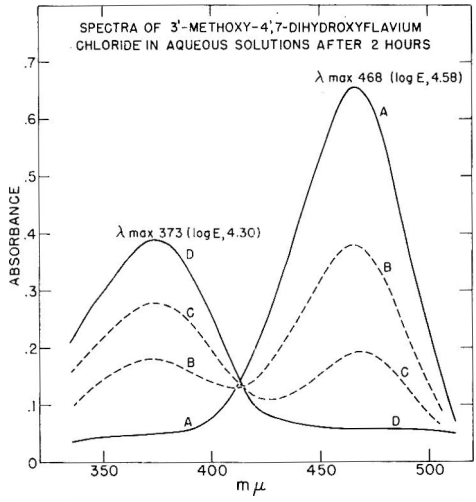


Fig. 2. Effect of pH on the spectra of 3′-methoxy-4′,7-dihydroxyflavylium chloride (5.2×10^{-3} g/L) at equilibrium in aqueous solutions: (A) pH 0.63, (B) pH 3.11, (C) pH 3.80, (D) pH 5.56.

and chalcone VIII forms in sulfite decoloration. Thus, a solution of VII in an aqueous buffer at pH 3.8 was allowed to reach equilibrium with the chalcone VIII (Fig. 3, spectrum A). Addition of sodium bisulfite to this solution caused immediate decoloration. The spectrum of the decolorized solution (Fig. 3, spectrum B), taken 1 min after the addition of bisulfite, shows that the flavylium salt ($\lambda_{max.}$ 468 mμ) has reacted completely whereas the chalcone ($\lambda_{max.}$ 373 mμ) concentration decreased only 13%. The reactive species, therefore, is not the chalcone but the flavylium ion VII, the small decrease in chalcone concentration presumably arising from its partial reconversion to the flavylium salt during this period.

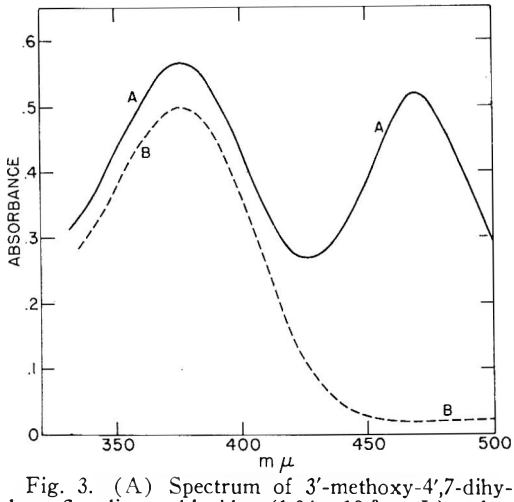


Fig. 3. (A) Spectrum of 3′-methoxy-4′,7-dihydroxyflavylium chloride (1.04×10^{-2} g/L) after standing 1 hr in aqueous solution at pH 3.8. (B) Sodium bisulfite (20 ppm) added to (A) and spectrum taken within 1 min.

The results with 3′-methoxy-4′,7-dihydroxyflavylium chloride were confirmed in a slightly different manner with 7-hydroxyflavylium chloride. This flavylium salt (Fig. 4, spectrum A) is predominantly converted

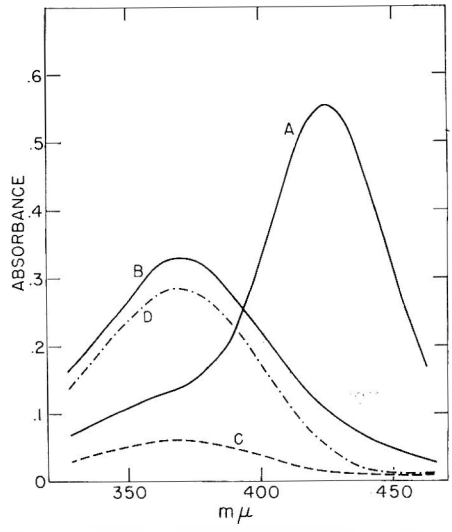
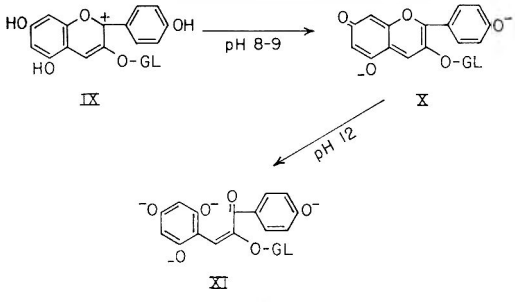


Fig. 4. Spectra of 7-hydroxyflavylium chloride (4.8×10^{-3} g/L) (A) in aqueous solution, pH 0.63 (B) in aqueous solution, pH 3.8, after 3 hr (C) sodium bisulfite (20 ppm) added to (B) and spectrum taken after 1 min (D) acid solution of the flavylium compound diluted with buffer, pH 3.8, containing sodium bisulfite (20 ppm) and spectrum taken after 1 min.

(86%) into the corresponding chalcone ($\lambda_{max.}$ 370) at equilibrium at pH 3.8 (Fig. 4, spectrum B). A strongly acid solution of the flavylium salt, diluted with buffer, pH 3.8, *containing* 20 ppm sodium bisulfite, is instantly decolorized (Fig. 4, spectrum C). If, however, the flavylium salt is diluted with buffer and allowed to stand until chalcone formation is complete and the bisulfite is then added, only a slow reaction ensues (Fig. 4, spectrum D).

Emphasis has been placed on the reversibility of sulfite decoloration in acid solutions. It is noteworthy, however, that the colorless sulfite compounds from natural and synthetic anthocyanins are also readily decomposed in alkaline solutions. The products recovered

in the alkaline solutions independently confirm the conclusions derived from the previous studies at pH 1–4. It is well known that in slightly alkaline (pH 8–10) solutions anthocyanins form highly colored ionized anhydro bases. In strongly alkaline (pH 12) solutions the ionized anhydro bases first formed, hydrolyze rapidly to fully ionized chalcones (Robertson and Robinson, 1928). Pelargonidin 3-glucoside IX, for example, treated with sodium bicarbonate (pH 8.3), formed the blue-red ionized anhydro base X, $\lambda_{max.}$ 552, 448 mμ. With NaOH this hydrolyzed rapidly to the yellow-brown ionized chalcone XI, $\lambda_{max.}$ ~ 420, 350 mμ.

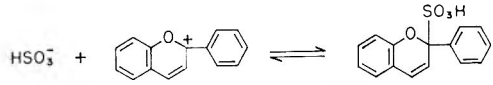


IX X XI

Sulfite decolorized pelargonidin 3-glucoside, treated with sodium bicarbonate and with sodium hydroxide under similar conditions, quantitatively formed identical products, viz., the ionized anhydro base and *then,* with NaOH, the fully ionized chalcone. It is clear that if the product formed in the sulfite decoloration of pelargonidin 3-glucoside had been a chalcone (XI)-bisulfite additive compound, the addition of sodium hydroxide would have resulted in *direct* formation of the ionized chalcone, without an ionized anhydro base intermediate.

Higher concentrations of bisulfite appreciably decolorize flavylium salts even in strongly acid solutions, e.g., 200 ppm of sodium bisulfite produced 85.6% decoloration of 3′-methoxy-4′,7-dihydroxyflavylium chloride at pH 0.63 in 15 min. It is highly unlikely, therefore, that a carbinol base of type II is involved in the decoloration. The experimental evidence indicates that sulfite decoloration of the natural and synthetic benzopyrylium compounds is dependent on the carbonium ion, bisulfite ion, and hydrogen ion concentrations. The following equilibrium reactions account satisfactorily for these observations:

(1) $HSO_3^- + H^+ \leftrightarrows H_ASO_3$



Thus, the slow and incomplete reaction at low pH is explained by a decrease in the bisulfite ion concentration, which counteracts the higher concentration of reactive flavylium carbonium ion at this pH.

REFERENCES

Charlesworth, E. H., J. J. Chavan, and R. Robinson. 1933. A synthesis of pyrylium salts of anthocyanidin type. XX. Morinidin chloride. Observations on cyanomaclurin. *J. Chem. Soc. London,* **1933**, 370.

Elstow, W. E., and B. C. Platt. 1950. The reduction of flavylium salts to flavans. *Chem. & Ind. London* **1950**, 824.

Freudenberg, K., H. Fikentscher, M. Harder, and O. Schmidt. 1925. Tannins and similar materials. XX. Transformation of cyanidin into catechin. *Liebig's Ann.,* **444**, 135.

Geissman, T. A. 1955. "Modern Methods of Plant Analyses." K. Paech and M. V. Tracey, eds. *Springer-Verlag,* 450.

Genevois, M. L. 1956. The isomeric keto-enol forms of anthocyanidins and anthocyanins. *Bull. Soc. Chim. Biol.* **38**, 7.

Jurd, L. 1963. Anthocyanins and related compounds. I. Structural transformations of flavylium salts in acidic solutions. *J. Org. Chem.* **28**, 987.

Jurd, L., and R. M. Horowitz. 1961. Spectral studies on flavonoid compounds. III. Polyhydroxychalcones. *J. Org. Chem.* **26**, 2561.

Perkin, W. H., R. Robinson, and M. R. Turner. 1908. The synthesis and constitution of certain pyranol salts related to brazilein and haematein. *J. Chem. Soc. London,* **93**, 1085.

Ribéreau-Gayon, P. 1959. Recherches sur les anthocyanins des vegetaux application au genre Vitis. Librairie Generale de L'enseignement, Paris.

Robertson, A., and R. Robinson. 1928. A synthesis of pyrylium salts of anthocyanidin type. XV. The synthesis of cyanidin chloride by means of *O*-benzoylphloroglucinaldehyde. *J. Chem. Soc. London* **1928**, 1526.

Sannié, C., and H. Sauvain. 1952. Edition du Museum, Paris, Quoted by Ribereau-Gayon, 1959.

Stadtman, E. R. 1948. Non-enzymatic browning in fruit products. *Advances in Food Research* **1**, 325.

Fatty Acid Composition as Influenced by Dietary Fatty Acids and Vitamin E Status in the Rabbit[a]

ROBERT F. BORGMAN
Clemson College, Clemson, S. C.

(Manuscript received July 21, 1963)

SUMMARY

Weanling New Zealand rabbits received a semipurified diet containing either semipurified oleic acid or semipurified linoleic acid, both with and without vitamin E. Rabbits receiving the oleic acid without vitamin E failed to develop skeletal muscle degeneration at 12 weeks, in comparison to marked degeneration at 8 weeks in the comparable group receiving linoleic acid. This would indicate that vitamin E requirements were very low when oleic acid was in the diet. Gallstones, composed largely of cholesterol, were plentiful in rabbits receiving oleic acid at 12 weeks, and a few were noted in rabbits receiving linoleic acid at 8 weeks. The gallstones were slightly more plentiful in females than males, but were not greatly influenced by the vitamin E status.

The influence of the dietary fatty acid upon the fatty acid composition of the liver, kidneys, heart, adipose deposits, skeletal muscle, and thoracic aorta was profound. The liver of rabbits receiving oleic acid was slightly less affected than the other organs, while the heart and adipose deposits of rabbits receiving linoleic acid were affected slightly more than the other organs. The sex did not appreciably influence the organ fatty acid percentages. Minor differences in fatty acid percentages were noted between rabbits receiving vitamin E and their deficient counterparts.

INTRODUCTION

In a previous study (Borgman, 1963), it was noted that the dietary fat, either lard or cod liver oil, influenced the fatty acid composition of the organs of rabbits, especially in the adipose deposits and skeletal muscle. The vitamin E status did not appear to influence the fatty acid composition of the organs, but skeletal muscle degeneration was more severe in vitamin-E-deficient rabbits receiving cod liver oil than those receiving lard. In the present study, it was of interest to determine the effect of specific semipurified fatty acids in the diet, i.e. oleic and linoleic, upon vitamin E metabolism in regard to skeletal muscle integrity and to determine their influence upon the fatty acid composition of several organs.

LITERATURE REVIEW

The prevention of peroxidation of polyunsaturated fatty acids *in vivo* has been indicated to be a major function of vitamin E (Horwitt *et al.*, 1956), and the biological damage resulting from avitaminosis E would, therefore, be expected to be proportional to the amount of polyunsaturated fatty acids in the diet. Lipid peroxidation has been observed in the heart, liver, and adrenals of vitamin-E-deficient rats fed large amounts of polyunsaturated fatty acids (Pritchard and Singh, 1960). Lipid peroxidation has also been detected in the liver mitochondria of vitamin-E-deficient rabbits (Zalkin and Tappel, 1960) and in the liver microsomes of vitamin-E-deficient rats (Tappel and Zalkin, 1960).

Skeletal muscle degeneration and creatinuria in vitamin-E-deficient rats were increased when cod liver oil was included in the diet, as compared with results when more saturated fats such as coconut oil and olive oil were in the diet (Century and Horwitt, 1960). This influence of dietary unsaturated fatty acids might be expected since the skeletal muscle fatty acids of rats fed corn oil, coconut oil, or cod liver oil closely

[a] From the Department of Food Technology and Human Nutrition, Clemson College, Clemson, South Carolina. Technical Contribution No. 450, South Carolina Agricultural Experiment Station. Published by permission of the Director.

This investigation was supported in part by grant No. A-4273 from the National Institute of Arthritis and Metabolic Diseases, Public Health Service.

Thanks are due Mrs. D. P. Rutherford, RN, for technical assistance.

resembled those in the diet (Century *et al.*, 1961).

EXPERIMENTAL METHODS

Weanling New Zealand rabbits, 3 weeks old, were maintained at constant room temperature with food and water *ad libitum*. The semipurified rabbit diet described by Wooley (1954) with the Briggs salt mixture (1952) was employed with the following changes in contents; 20% semipurified fatty acid substituted for corn oil, corn starch reduced to 7.3%, and α-tocopherol acetate increased to 10 mg per 100 g of diet. Analyses of the semipurified fatty acids are listed at the top of Table 2. The completed diets were mixed in amounts sufficient for 5 days and kept under refrigeration to minimize deterioration. Rabbits were randomly assigned by sex with littermates spread between groups into 4 groups containing 3 males and 3 females each. Treatments were as follows: I, oleic acid with vitamin E; II, oleic acid without vitamin E; III, linoleic acid with vitamin E; and IV, linoleic acid without vitamin E. Rabbits receiving oleic acid were continued for 12 weeks, while those receiving linoleic acid were terminated at 8 weeks because of marked skeletal muscle degeneration in group IV.

The semipurified oleic acid was obtained from Distillation Products, Rochester, New York, and contained 1.7 mg vitamin E per 100 g, when analyzed by the method of Bieri *et al.* (1961). The semipurified linoleic acid was obtained from Nutritional Biochemicals Company, Cleveland, Ohio, and contained 1.8 mg vitamin E per 100 g, when analyzed by the same method.

Rabbits were sacrificed by exsanguination, and the heart, liver, kidneys, adipose deposits forming the kidney pads, a section of the semimembranosus, and the thoracic aorta were removed. All but the adipose deposits were trimmed of visible fat, partially sliced into 5-mm sections, dried to a constant weight under vacuum, and finally extracted with petroleum ether in the Soxhlet apparatus for 24 hr. Methyl esters of fatty acids were formed in the dried ether extract by the method described by Stoffel *et al.* (1959), and separation and detection made by gas-liquid chromatography with a 10-ft-¼-in. diethylene glycol succinate column and a thermal conductivity detector. Samples from 1 to 3 μl were used with the column temperature at 190°C and the helium flow rate at 100 ml per min. Identifications were made by graphing the log of the retention times, employing a silicone column to verify chain lengths, and by the addition of small amounts of standards to the samples, i.e. C14, C16, C16:1, C18, C18:1, C18:2, C18:3, C20, and C20:4. Percentages of fatty acids were computed by a disc-type integrator. No attempts were made to identify the positions of double bonds.

A portion of the semimembranosus was preserved in buffered formalin until slides were prepared by the hematoxylin eosin technique.

Statistical analyses were performed by analysis of variance technique (Snedecor, 1946).

RESULTS

Rabbits receiving oleic acid remained apparently normal during the 12-week trial period, regardless of vitamin E status, whereas rabbits receiving linoleic acid without vitamin E evidenced muscular weakness and general debility at 8 weeks. Weight gains (Table 1) were greater in rabbits receiving oleic acid than in those receiving linoleic acid, but were not consistently influenced by vitamin E status.

Skeletal muscle degeneration (Table 1) was very slight to absent in all rabbits receiving oleic

Table 1. Body weight, skeletal muscle, and gall bladder observations.

Group and treatment	Weeks on trial	Av. weekly wt. gain (g) 0–8 wk	Av. weekly wt. gain (g) 8–12 wk	Evaluation of muscle degeneration [a]	Incidence of gallstones (3 rabbits each sex)
I) Oleic acid w/vit. E	12				
Males		128	31	0–v. sl.	2—1 sl. & 1 mod.
Females		101	18	0–v. sl.	2—1 sl. & 1 sev.
II) Oleic acid w/o vit. E.	12				
Males		86	55	0	3—1 sl. & 2 mod.
Females		99	38	0	3—1 sl., 1 mod. & 1 sev.
III) Linoleic acid w/vit. E	8				
Males		59	....	0–v. sl.	0
Females		62	....	0–v. sl.	1 sl.
IV) Linoleic acid w/o vit. E	8				
Males		52	....	mod.–sev.	0
Females		55	....	mod.–v. sev.	1 sl.

[a] Several sections were taken from each semimembranosus and evaluations made as described previously (Borgman and Underbjerg, 1962). Very slight degeneration is not specific for avitaminosis E.

Table 2. Averages of fatty acid percentages in organs.

Treatment and group	Fatty acids [a]							
	C14	C14:1	C16	C16:1	C18	C18:1	C18:2	C18:3
Semipurified oleic acid in diet	3.2	2.7	3.9	14.0	0	62.1	11.4	1.1
Semipurified linoleic acid in diet	0	0	6.7	1.8	0	11.0	75.5	5.1
Heart								
Weanling males [b]	4.0	1.9	21.4	5.4	9.2	21.1	16.8	2.0
I–Oleic acid w/vit. E	1.9	1.2	6.1	11.6	1.5	64.2	9.8	2.0
II–Oleic acid w/o vit. E	1.8	1.2	4.9	11.7	1.2	67.3	7.2	2.2
III–Linoleic acid w/vit. E	0.3	0.1	8.2	1.3	2.6	10.0	73.8	3.0
IV–Linoleic acid w/o vit. E	0.5	0.2	11.0	2.0	2.4	9.5	70.5	2.9
Liver								
Weanling males [b]	1.5	0.9	22.2	7.8	13.1	22.8	21.1	3.0
I (groups as above)	0.4	0.2	8.7	8.6	5.6	58.4	13.5	2.2
II	1.3	0.8*	7.2	10.4	3.4	53.5	16.9	3.8
III	0.2	t	10.4	1.6	5.1	13.3	63.8*	2.8
IV	0.2	0.1	13.9	2.3	0.3	20.2*	58.6	3.9
Kidneys								
Weanling males [b]	4.1	4.0	19.1	5.9	9.9	20.4	18.4	4.5
I	1.6	1.4	5.9	10.9	1.8	64.6	8.2	3.2
II	2.2	1.3	5.4	13.0	1.5	63.3	6.4	3.2
III	0.4	0.8*	8.0	4.0	2.4	12.5	65.8	2.7
IV	0.4	0.2	9.0	3.0	1.4	13.8	63.2	4.8*
Adipose deposits								
Weanling males [b]	4.2	1.9	25.5	5.7	9.7	19.9	20.1	2.5
I	2.5	1.6	6.0	11.2	1.4	67.4*	5.4	2.6
II	2.4	1.8	5.0	11.4	1.0	61.6	10.4	3.1
III	0.6	0.2	9.5	2.6	1.2	11.3	71.7	2.2
IV	0.3	0.2	12.0	1.3	0.8	11.4	68.2	3.7
Skeletal muscle								
Weanling males [b]	3.4	2.5	25.5	5.4	10.8	19.2	20.3	3.3
I	2.8	1.8	7.8	13.0	2.0	63.1	5.8	1.8
II	2.6	1.8	6.2	12.2	1.8	62.4	7.2	2.8
III	0.4	t	10.8	2.0	2.4	11.6	66.4*	4.4
IV	0.1	0.1	14.6	2.9	2.7	13.8	61.0	3.4
Thoracic aorta								
Weanling males [b]	5.1	4.6	30.8	6.8	6.4	21.0	10.4	2.2
I	2.6	1.6	6.9	10.6	1.9	64.2	6.0	1.6
II	2.4	1.4	6.2	11.4	1.7	62.4	7.0	4.9*
III	0.8	0.4	11.4	2.4	1.3	14.2	64.7	3.9
IV	0.5	0.2	12.0	2.4	1.4	14.0	62.5	4.1

t = indicates values under 0.1%.

* These values were significantly higher ($p < 0.05$) than the corresponding group receiving the same fatty acid, but a different vitamin E status.

[a] The fatty acid percentages are averages of the rabbits in each group. The values for the sexes were combined since only a very few of the values were different statistically at the $p < 0.05$ level while none were significant at the $p > 0.01$ level. The dietary fatty acid significantly ($p < 0.01$) influenced the percentages in all organs except for C18:3. Only traces of C20:4 were noted in rabbit tissues. The weanling rabbits had shorter chain fatty acids, C8 through C12:1, in their tissues; but these were almost completely absent in the rabbits on trial. Very small unidentified peaks were usually noted between C14:1 and C16 in the oleic acid and in the tissues of groups I and II. Small unidentified peaks were usually found between C16:1 and C18 in the oleic acid and the tissues of all groups.

[b] The percentages of weanling rabbits are included for reference and were obtained in a previous study (Borgman, 1963).

acid, with or without vitamin E. This was a surprising observation for rabbits not receiving vitamin E, but vitamin E requirements were perhaps low when the rabbits received oleic acid, and the amount of vitamin E in the oleic acid was sufficient for muscle metabolism. Rabbits receiving linoleic acid with vitamin E had little muscle pathology, compared to those not receiving vitamin E. It is possible that the very slight degeneration noted in rabbits receiving vitamin E and either oleic or linoleic acid was the result of a diet containing a relatively high fat content.

Gallstones were observed in the rabbits receiving oleic acid (Table 1), and were slightly more prevalent in the females than the males. Rabbits receiving linoleic acid also had a few stones, but these were on trial for only 8 weeks, so no direct comparison between oleic acid and linoleic acid was possible. Vitamin E status did not appear to affect the incidence of gallstones. Preliminary analyses of the gallstones indicated that they contained 35–50% cholesterol on a dry-weight basis. A residue, 10% of the dry weight, remained after ashing in a muffle furnace. A more detailed study of the composition is in progress.

Dietary fatty acid had a profound influence on organ fatty acid percentages (Table 2) in all groups, although the time on trial may have confounded the results since rabbits receiving oleic acid were continued 12 weeks and those receiving linoleic acid were continued 8 weeks. In rabbits receiving oleic acid, the liver was affected only slightly less than other organs by the dietary fatty acid, and the level of C18:2 in the organs of these rabbits was surprisingly low. In rabbits receiving linoleic acid, the heart and adipose deposits were influenced to a slightly greater degree than the other organs examined. Sex did not appear to influence organ fatty acid percentages appreciably. The influence of vitamin E status was not marked. In rabbits given oleic acid with vitamin E, C14:1 was lower in the liver, C18:1 higher in the adipose deposits, and C18:3 lower in the thoracic aorta than in the vitamin-E-deficient counterparts. In the rabbits given linoleic acid with vitamin E, C18:1 was lower and C18:2 higher in the liver, C14:1 higher and C18:3 lower in the kidneys, and C18:2 higher in the skeletal muscle than in the corresponding vitamin-E-deficient group.

DISCUSSION

Apparently the two double bonds in linoleic acid had a pronounced effect upon vitamin E requirements, as evidenced by the marked pathology in the deficient rabbits receiving linoleic acid when compared to the absence of pathology in those receiving oleic acid. The amount of vitamin E required to prevent chick encephalomalacia was increased when corn oil, high in linoleic acid, was added to the diet (Century and Horwitt, 1958; Horwitt *et al.*, 1959), and the peroxide erythrocyte hemolysis reaction in vitamin-E-deficient rats was greater when the linoleic acid content of the erythrocytes was increased by dietary means (Alfin-Slater *et al.*, 1961). Vitamin E requirements of infants were increased when the diet was high in linoleates (Asfour *et al.*, 1963).

Dietary fatty acids had a profound effect upon the composition of the various organs, but neither the sex nor vitamin E status of the rabbits had such a marked effect. However, Pritchard and Singh (1960) noted a reduction of polyunsaturated fatty acid concentrations in the heart, liver, adrenals, and plasma of vitamin-E-deficient rats. Hove and Seibold (1955) found less linoleic, arachidonic, and pentaenoic acids in muscle fat, and less linoleic and pentaenoic acids and more oleic acid in the liver, of vitamin-E-deficient swine. Some tendencies in this direction were noted in vitamin-E-deficient rabbits.

REFERENCES

Alfin-Slater, R. B., H. W. Hansen, and R. L. Shull. 1961. Relationship of dietary linoleate to tocopherol. *Federation Proc.* **20**, 366.

Asfour, R., S. A. Hashim, G. Schuttringer, J. I. Maraca, W. H. Sebrell, and T. B. Van Itallie. 1963. Effect of linoleate-rich diet on tocopherol requirement in infants. *Federation Proc.* **22**, 550.

Bieri, J. G., C. J. Pollard, I. Prange, and H. Dam. 1961. The determination of α-tocopherol in animal tissues by column chromatography. *Acta Chem. Scand.* **15**, 783.

Borgman, R. F. 1963. Fat metabolism as influenced by dietary fats and avitaminosis E. *Am. J. Vet. Research.* In press.

Borgman, R. F., and G. K. L. Underbjerg. 1962. The influence of adrenal cortical hormone, parathyroid hormone, and thyroparathyroidectomy on avitaminosis E in rabbits. *Am. J. Vet. Research* **23**, 115.

Briggs, G. M., M. R. Spivey, J. C. Keresytesy, and M. Silverman. 1952. Activity of citrovorium factor for the chick. *Proc. Soc. Exptl. Biol. Med.* **81**, 113.

Century, B., and M. K. Horwitt. 1958. Some factors in production of encephalomalacia in

the vitamin E deficient chick. *Federation Proc.* **17**, 1854.

Century, B., and M. K. Horwitt. 1960. Role of diet lipids in the appearance of dystrophy and creatinuria in the vitamin E-deficient rat. *J. Nutrition* **72**, 357.

Century, B., L. A. Witting, C. C. Harvey, and M. K. Horwitt. 1961. Changes in fatty acid composition in skeletal muscle of rats induced by varying the lipid in the diet. *Federation Proc.* **20**, 366.

Horwitt, M. K., C. C. Harvey, G. D. Duncan, and W. C. Wilson. 1956. Effect of limited tocopherol intake in man with relationships to erythrocyte hemolysis and lipid oxidations. *Am. J. Clin. Nutrition* **4**, 408.

Horwitt, M. K., C. C. Harvey, and B. Century. 1959. Effect of dietary fats on fatty acid composition of human erythrocytes and chick cerebella. *Science* **130**, 917.

Hove, E. L., and H. R. Seibold. 1955. Liver necrosis and altered fat composition in vitamin E-deficient swine. *J. Nutrition* **56**, 173.

Pritchard, E. T., and H. Singh. 1960. Lipid peroxidation in tissues of vitamin E deficient rats. *Biochem. & Biophysical Research Comm.* **2**, 184.

Snedecor, G. W. 1946. "Statistical Methods." Iowa State College Press, Ames, Iowa.

Stoffel, W., F. Chu, and E. H. Ahrens, Jr. 1959. Analysis of long-chain fatty acids by gas-liquid chromatography. Micromethod or preparation of methyl esters. *Anal. Chem.* **31**, 307.

Tappel, A. L., and H. Zalkin. 1960. Inhibition of lipid peroxidation in microsomes by vitamin E. *Nature* **185**, 35.

Wooley, J. G. 1954. Growth of three to four week old rabbits fed purified and stock rations. *J. Nutrition* **52**, 39.

Zalkin, H., and A. L. Tappel. 1960. Studies of the mechanism of vitamin E action. IV. Lipide peroxidation in the vitamin E-deficient rabbit. *Arch. Biochem. Biophys.* **88**, 113.

Isolation of Beta-Elemene from Orange Oil

G. L. K. HUNTER AND G. L. PARKS
Fruit and Vegetable Products Laboratory,[a] Winter Haven, Florida

(Manuscript received August 31, 1963)

During an investigation of Valencia cold-pressed orange oil, a sesquiterpene hydrocarbon was isolated from this source for the first time. The compound, which was obtained from the oil by distillation, column chromatography, and gas chromatography, was identified as β-elemene.

Orange oil was distilled on a semimicro spinning band column, and the fraction boiling between 110 and 120°C/9 mm was chromatographed on a 1-inch × 2-ft column containing a 50:50 mixture of silicic acid–cellulose. The material eluting with *n*-hexane was collected. The residue, upon evaporation of the solvent, was gas chromatographed on a ¼ in. × 10-ft column containing 30% carbowax 20M on Chromosorb P. The column was operated at 150°C with a helium flow rate of 60 cc/min. The material represented by the third peak in Fig. 1 was due to ylangene, reported by Teranishi *et al.* (1963). The material represented by the fourth peak in Fig. 1 was trapped, and the infrared curve obtained on the substance is shown in Fig. 2. The material had a molecular weight of 204 by mass spectroscopy, indicating the sesquiterpene structure having an empirical formula of $C_{15}H_{24}$. The sesquiterpene was reduced to the saturated parent structure with platinum oxide in ethanol, resulting in a molecular weight increase of 6 to 210. The uptake of three moles of hydrogen showed the presence of three double bonds. The infrared spectrum indicated at least one terminal double bond by its absorption at 890 cm^{-1}, and at least one vinyl group by its absorptions at 910 cm^{-1} and 1000 cm^{-1} on a hydrocarbon structure (Bellamy, 1958). The peak at 790 cm^{-1} is due to an impurity that has been shown to decrease by repeated gas chromatography. The ultraviolet spectrum showed no absorption in the region between 3500 and 2100 A, indicating a hydrocarbon with three nonconjugated terminal double bonds. Nuclear magnetic-resonance (NMR) spectra show seven vinyl protons, one singlet methyl at 1.0 ppm, and two vinylic methyl groups at 1.5 ppm and 1.7 ppm. The NMR of β-elemene was obtained in deuterated chloroform with tetramethylsilane as the internal reference. The β-elemene structure was indicated and conclusively established by comparison of the infrared curve with that published by Asselineau and Asselineau (1957).

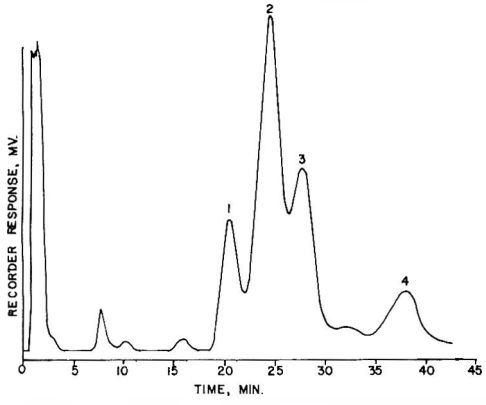


Fig. 1. Chromatogram of β-elemene from orange oil, (1) octylacetate, (2) decanal, (3) ylangene, (4) β-elemene.

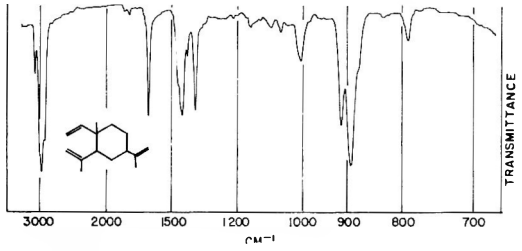


Fig. 2. Infrared spectrum of β-elemene.

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

Mention of brand names is for identification only, and does not imply recommendation.

ACKNOWLEDGMENT

The authors thank Dr. Werner Herz, Florida State University, Tallahassee, Florida, for carrying out the nuclear magnetic-resonance study.

REFERENCES

Asselineau, Cecile, and Jean Asselineau. 1957. Sur la composition de l'huile de racine d'aunee. Etude de la fraction insaponifiable. *Bull. Soc. Chim. France.* No. 11–12, p. 1359.

Bellamy, L. J. 1958. "The Infrared Spectra of Complex Molecules." p. 34. John Wiley and Sons, Inc., New York, N. Y.

Teranishi, Roy, T. M. Schultz, W. H. McFadden, R. E. Lundin, and D. R. Black. 1963. Volatiles from orange. Hydrocarbons identified by infrared, nuclear magnetic resonance, and mass spectra. *J. Food Sci.* **28**, 541.

ERRATUM

Journal of Food Science, Vol. 28, No. 4, 1963. "Determination of Chlorophylls, Chlorophyllides, Pheophytins and Pheophorbides in Plant Material," by R. C. White, I. D. Jones and Eleanor Gibbs.

Page 434 change equation (1) to read: $Py_at + Po_at = \ldots$. rather than $Py_a + Po_at = \ldots$.

Page 434 change equation (2) to read: $Py_bt + Po_bt = \ldots$. rather than $Py_b + Po_bt = \ldots$.

The Antioxidant Activity of Vegetable Extracts I. Flavone Aglycones[a]

DAN E. PRATT[b] AND BETTY M. WATTS
Department of Food and Nutrition, Florida State University
Tallahassee, Florida

(Manuscript received May 28, 1963)

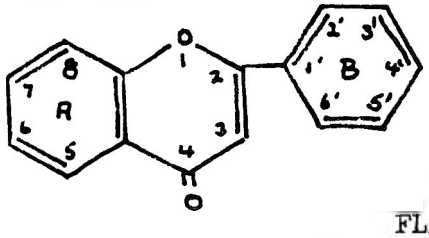
SUMMARY

Hot-water extracts of several plant tissues showed the following descending order of antioxidant activity, both as cover solutions of meats and in artificial systems: green onion tops, green pepper seeds, green peppers, celery, potato peels, green onions, and tomato peel. The over-all antioxidant activity of extracts was not changed by hydrolysis of flavone glycosides, indicating that the naturally occurring glycosides possessed the same activity as the aglycones. The antioxidant activities of several known aglycones were compared and related to structural characteristics. Quercetin derivatives were the principal flavones of the extracts studied. Quercetin isolated from hydrolyzed extracts accounted for a large portion, but not all, of the antioxidant activity of the extracts. Because of their effectiveness in heme-catalyzed systems, the most significant role of the extracts as antioxidants is apparently their ability to break the chain reaction in lipid oxidation.

Workers in this laboratory (Lewis and Watts, 1958; Cofer, 1963; Ramsey, 1962) have shown that some hot-water vegetable extracts are effective in retarding lipid oxidation. Cofer (1963) screened approximately 40 vegetable extracts and found that antioxidant activity varied widely; however, nearly all extracts tested possessed some antioxidant activity. Ramsey (1962) studied six vegetable extracts as antioxidants in heme- and copper-catalyzed systems. The tested extracts possessed the ability to chelate copper as well as to provide protection in heme-catalyzed lipid oxidation systems. In the above studies, unfractionated vegetable extracts were examined.

Hot-water extracts of plant tissues are complex mixtures containing several classes of compounds that might contribute to their antioxidant capacity. In addition to phenols, aromatic amines, sulfhydryl compounds and others may function as primary antioxidants. Ascorbic and citric acids may contribute to antioxidant activity as synergists.

Flavonoids, a major group of plant phenols, include compounds that are potent antioxidants. The antioxidant ability of flavonols (3-hydroxy flavone derivatives) has been well established in both non-aqueous lipid systems (Richardson *et al.*, 1947; Kurth and Chan, 1951; Heimann *et al.*, 1953; Simpson and Uri, 1956; Mehta and Seshadri, 1959) and in aqueous-lipid systems (Simpson and Uri, 1956; Kelly and Watts, 1957).



FLAVONE

Antioxidant activity of flavonols is apparently due to their ability to act as free radical acceptors (Heimann *et al.*, 1953; Simpson and Uri, 1956; Lea, 1958; Mehta and Seshadri, 1959; Crawford *et al.*, 1961) and/or to complex metal ions (Lewis and Watts, 1958; Kelley and Watts, 1957; Mehta and Seshadri, 1959). Heimann and Reiff (1953) studied flavone derivatives as antioxidants

[a] Presented at the 23rd Annual Meeting of the Institute of Food Technologists, Detroit, Michigan, May 28, 1963.

[b] Present address: Department of Foods and Nutrition, University of Wisconsin, Madison, Wisconsin.

Thanks are expressed to the S. B. Penick Co., New York, for gift samples of flavonoids.

in ethyl linoleate and concluded that the following structural characteristics were correlated with antioxidant activity: 1) the 2-3 olefin group and the ketone in position 4 of the pyrone ring; 2) 3-hydroxylation; 3) ortho hydroxylation of the B ring; 4) meta hydroxylation of the chromone system (i.e., 5,7-dihydroxy). The first three reportedly increased activity whereas the latter had an adverse effect.

The factors considered by Heimann and Reiff (1953) to be involved in antioxidant activity have been considered by other workers (Kurth and Chan, 1951; Simpson and Uri, 1956; Mehta and Seshadri, 1959; Crawford *et al.*, 1961; Lea, 1962). Considerable confusion exists as to the importance of 5,7-meta-hydroxylation, the 2,3 double bond, and 3-hydroxylation. Mehta and Seshadri (1959) found that 5,7-meta-hydroxylation did not decrease antioxidant activity. Kurth and Chan (1951) and Crawford *et al.* (1962) found dihydroquercetin to be an effective antioxidant, thus minimizing the importance of the 2,3 double bond. Crawford *et al.* (1961) also found that methylation of the 3-hydroxyl group of quercetin lowered antioxidant activity only slightly. In a recent review, however, Lea (1962) reported that work in his laboratory demonstrated that methylating the 3-hydroxyl group or hydrogenating the 2,3 double bond greatly reduced the effectiveness of polyhydroxyflavones as antioxidants.

Flavones occur in nature mainly as glycosides, which may be hydrolyzed by acid-heat treatments to form aglycones and sugars. Flavone aglycones are readily separated by paper chromatography using water as the developing solvent. The flavone aglycones do not move in water development, whereas the glycosides and non-planar aglycones (flavonoids without the 2,3 double bond, i.e., flavanones, flavanonols, and catechins) travel appreciably (Bate-Smith, 1948; Harborne, 1959; Seikel, 1962). Free amino acids and sugars also move with the solvent front (Seikel, 1962).

The present study was designed: 1) to investigate the use of hot-water vegetable extracts in retarding lipid oxidation in aqueous-lipid systems, 2) to isolate and identify flavone aglycones of the extracts and ascertain their effectiveness as lipid antioxidants, and 3) to correlate structural features with antioxidant activity.

EXPERIMENTAL

Preparation of extracts. Vegetables were purchased at local retail markets. All vegetables and equipment were thoroughly washed and rinsed several times with distilled water. The potatoes used were white varieties of the previous year's crop. Tomatoes were firm ripe. The peels of the potatoes and tomatoes were obtained by hand peeling with a stainless-steel knife; approximately 1–2 mm of vegetable flesh remained on the peels.

Hot-water extracts of vegetables were prepared by adding one part of finely chopped vegetable tissue to five parts of boiling distilled water; on returning to a boil the mixture was heated 5 min. The vegetable residue was removed by filtration. To facilitate subsequent hydrolysis and chromatographic treatments, the extracts were concentrated by boiling to 20% of the volume of the water used for extraction. When reconstituted to the original volume, these concentrates did not differ significantly in antioxidant activity from the original extracts. Herein the "percentage extract" is used to denote the grams of plant tissue to prepare 100 ml of extract solution.

Hydrolysis of extracts. Hydrolysis methods for flavonoid glycosides described in the literature vary widely in concentration of acid and heating conditions. Unfortunately, evidence for the effectiveness of the procedures used has not generally been included. With hydrolysis in 0.5*N* HCl at 100°C for 30 min, the maximum aglycone concentration could be separated from the extracts, and quantitative yields of aglycones from standards were greatest. These hydrolysis conditions were used by Jurd *et al.* (1957). As shown in Table 1, the aglycone recovery was approximately 100%

Table 1. Effect of acid concentration on glycoside hydrolysis (30 min at 100°C).

Glycoside	*N* HCl	Recovery of quercetin: Concentration[a] $\times 10^{-5}M$	Recovery of quercetin: %
Quercitrin	0.1	0.68	41
	0.25	0.96	59
	0.5	1.68	102
	1.0	1.32	81
	1.5	1.00	61
Rutin	0.1	0.88	54
	0.25	1.38	84
	0.5	1.60	98
	1.0	1.48	90
	1.5	1.32	81

[a] Initial glycoside concentration $1.64 \times 10^{-5}M$.

following hydrolysis. With lower concentration of acid, hydrolysis was not complete, and further degradation of the molecules apparently occurred when more severe conditions were employed.

For comparisons of hydrolyzed and unhydrolyzed extracts, the concentrated extracts were divided into two portions. One portion was hydrolyzed by making 0.5*N* in respect to HCl and heating 30 min at 100°C. Following hydrolysis the extracts were neutralized with NaOH. Aliquots of the hydrolyzed and unhydrolyzed extracts were adjusted to 20% for use in antioxidant studies.

Chromatographic fractionation of extracts. Flavonoids of hydrolyzed and unhydrolyzed extracts were separated by paper chromatographic techniques. Generally, square sheets (46.5 cm) of Whatman No. 3MM filter paper were used. The concentrated extracts were alternately streaked and dried until a total representing 4 g of vegetable had been deposited three inches from one edge of the paper. In the initial separation, descending chromatography was used with water as the developing solvent. The chromatograms were irrigated until the solvent front had migrated approximately 30 cm. In this system the flavone aglycones remained essentially at the origin, while glycosides, non-planar flavonoid aglycones, free amino acids, sugars, and other water-soluble components moved in the solvent.

The water-developed chromatograms were cut into three bands: 1) the origin, including approximately 5 cm of the developed chromatogram (band A); 2) the next 15 cm of developed area (band B); and 3) the section adjacent to the solvent front, including approximately 10 cm of the chromatogram (band C). The bands were eluted by three extractions in boiling water. Spectrophotometric comparison of the eluates with fractions eluted in boiling methanol and u.v. examination of the chromatographic paper following elution demonstrated that elution was complete. Eluted fractions to be used for antioxidant studies were diluted so that the concentration represented that which would be present in a 20% extract of the original vegetable.

To separate flavone aglycones and remove possible contaminating material, the A fractions were streaked and chromatographed using the upper layer of a 4:1:5 two-phase mixture of 1-butanol-acetic acid-water (BAW).

Identification of aglycones. The aglycones were identified by comparing chromatographic R_f values and u.v. spectral curves with known compounds. Two-dimension chromatographic techniques were used in identification studies. The solvents included BAW, 15% acetic acid, 80% phenol, and water. Spectral analyses were carried out in absolute ethanol using a Bausch and Lomb "505" recording spectrophotometer for obtaining absorption curves and a Beckman DU spectrophotometer for quantitative absorbance data at established peaks.

Tests of antioxidant activity. The antioxidant effectiveness of the extracts, isolated compounds, and known standards was ascertained by determining: 1) the malonaldehyde concentration of beef slices covered with test solutions, and 2) the bleaching time of carotene-lard solutions in contact with the test solutions.

For the former determination, eye round of beef was used. External fat was trimmed from the surface of the roast and the beef was roasted to an internal temperature of 74°C in an oven preheated to 149°C. The roasted beef was mechanically sliced to a thickness of approximately 3 mm.

Thirty-five grams of beef slices were placed immediately in 100-ml beakers and covered with 35 ml of test solution, covered with aluminum foil, and stored at approximately 3°C in a refrigerator. At specified intervals, samples were removed, the cover solution decanted, and thiobarbituric (TBA) tests run on the beef slices. For the TBA tests, 30 g of the meat were blended with 60 g of water, and 30 portions (10 g of beef) of the blended materials were used for each analysis. The distillation technique reported by Tarladgis *et al.* (1960) was employed.

The second means of determining the antioxidant effectiveness of the test materials was by two modifications of a method reported by Scarborough and Watts (1959). Initially, 1.0 ml of test solution was absorbed onto a 7.5-cm filter-paper disc in a Petri dish. A second filter disc (7.0 cm in diameter) was dipped into melted lard containing 0.01% carotene and placed over the first, eliminating air bubbles from the system. The Petri dishes were sealed with rubber sleeves and stored in an incubator at 55°C. The fat diffused through both papers, thus coming into immediate contact with the aqueous phase. Peroxide formation was accompanied by bleaching of the carotene. Visual observations were made at 12-hour intervals. The ratio of time required for the sample to half-bleach to time required for the control to half-bleach was considered the antioxidant index.

To test small amounts of extract fractions, a second modification of the carotene-lard system was used. Four discs, 12 mm in diameter, were placed on a 7.5-cm filter paper, saturated with the carotene-lard solution in a Petri dish. To each disc 0.05 ml of test solution was added. The Petri dishes were sealed with rubber sleeves and stored at 38°C. As before, the time required for the discs to half-bleach was used to calculate the antioxidant index.

RESULTS AND DISCUSSION

Antioxidant activity of vegetable extracts by two different methods. The data in Table 2 show that the two methods used to evaluate antioxidant activity are in very good agreement. The hot-water extracts varied widely in their ability as antioxidants in lipid-aqueous systems. The effectiveness of the extracts in retarding lipid oxidation of the beef slices, which is a heme-catalyzed reaction, indicate that the principal antioxidant activity is that of free radical acceptors. The antioxidant components of the extracts cannot be envisaged as chelating the iron of heme compounds.

Table 2. Antioxidant activity of vegetable extracts.

Extract	TBA no. of roast beef slices at 3°C		Antioxidant index carotene-lard system at 45°C
	3 days	6 days	
Control	8.6	12.4	1.0
20% tomato peel	8.4	10.2	1.0
20% green onion	4.0	4.7	6.0
20% potato peel	3.6	4.3	7.5
20% celery	3.2	4.1	7.5
20% green pepper	2.4	3.9	9.0
20% green pepper seed	1.7	3.2	>12.5
20% green onion top	1.1	2.4	>12.5
50% green onion top	0.1	—	—

An interesting observation is that extracts from inedible portions of the plants had greater protective effect than those from the edible portion. Extracts of green onion top had greater activity than extracts of the onions; green-pepper-seed extracts were more effective than green-pepper extracts, and potato-peel extracts gave greater protection than extracts from potatoes. In another experiment, celery leaves had greater antioxidant capacity than celery stalks. Flavonoids have been found to occur more abundantly in inedible portions of a number of plants (Bate-Smith, 1954; 1959; Swain, 1962).

The antioxidant activity of the extracts, except for tomato peels, agrees with other observations in this laboratory (Cofer, 1963). Cofer found 20% tomato-peel extracts to have an antioxidant index of two, whereas tomato peels in the present study were void of antioxidant activity. Wu and Burrell (1958) studied three varieties of tomatoes, and found considerable concentrations of flavonoids (quercitrin, dutin, and naringenin) in the peel but not in the flesh. The flavonoid concentrations of the tomatoes used in this experiment were extremely low, and were not investigated further.

Hillis and Swain (1959) pointed out that concentrations of flavonoid may change markedly during maturation and storage. Kefford (1959) reported qualitative variations related to cultural varieties.

In using the unfractionated vegetable extracts as antioxidants for meat, typical vegetable odors were usually detectable in the beef samples. Green-pepper-seed extracts imparted a pungent odor resembling that of acrolein.

Antioxidant activity of hydrolyzed and unhydrolyzed extracts. The effect of hydrolysis of extracts on antioxidant activity is shown in Table 3. Hydrolysis had no apparent effect on over-all activity of the extracts. The activity of fractions separated by paper chromatography with water as the chromatographic solvent was considerably changed, however, after hydrolysis.

Fraction A, which did not move in the water solvent, includes the flavone aglycones.

Table 3. Effect of hydrolysis of extracts on antioxidant activity.

Extract (20%)	Antoxidant indices							
	Unhydrolyzed				Hydrolyzed			
	Extract	A	B	C	Extract	A	B	C
Potato	1.1	0.9	1.0	1.0	1.2	1.2	1.0	0.9
Green onion	5.2	1.9	4.0	2.2	5.0	3.8	3.2	1.5
Green pepper	5.5	1.8	4.3	2.7	5.2	4.0	2.3	1.9
Potato peel	>7.2[a]	1.8	6.9	4.6	>7.2[a]	4.3	6.5	2.0
Green onion top	>7.2[a]	3.8	5.5	5.0	>7.2[a]	7.0	4.5	2.5
Green pepper seed	>6.5	4.2	5.1	4.7	>6.5[a]	>6.5[a]	3.9	2.5

[a] Experiment discontinued because of mold growth in Petri dishes.

Eluates from the A band were devoid of vegetable odors as well as amino acids and sugars. Except for the potato extract, which did not possess any significant antioxidant effect, hydrolysis markedly increased the activity of the A fractions. The activity of the B fractions decreased slightly from hydrolysis, and the C fraction activity decreased considerably. The increase in A fraction activity and the decrease in activity of the other fractions demonstrate that hydrolyzable components are responsible for a considerable amount of the antioxidant activity of the extracts. Coupled with the fact that hydrolysis did not alter the activity, this indicates that the glycosides and aglycones of the extracts possessed approximately the same activity.

Kelley and Watts (1957), using a carotene-lard system similar to the ones used in this study, also found that 3-glycosides of quercetin had approximately the same protection as quercetin. Crawford *et al.* (1961) found that methylation of the 3-hydroxyl group lowered the antioxidant capacity of quercetin only slightly. However, considering the importance attached to the free 3-hydroxyl by others (Heimann and Reiff, 1953; Simpson and Uri, 1956; Lea and Swoboda, 1956; Mehta and Seshadri, 1959; Lea, 1962), the reason that the 3-glycosides have the same activity as the aglycones is not readily apparent.

Bands B and C still possessed appreciable antioxidant activity after hydrolysis. This means that vegetable components other than flavones are also important. The authors are currently studying the identity of these components.

Identification of aglycones. Chromatographic and u.v. spectral analyses of aglycones isolated from the A fractions of hydrolyzed extracts are summarized in Table 4. Chromatographic studies failed to demonstrate the presence of compounds other than flavonol aglycones in the A band. Isolated and standard aglycones exhibited nearly identical u.v. absorbance spectra in absolute ethanol.

Quercetin derivatives are the principal flavones of green onions, green onion tops, green peppers, green pepper seeds, and potato peels. Only in green onion tops was a second flavone aglycone, myricetin, detected. The myricetin concentration in the several green-onion-top extracts analyzed was approximately one-tenth the concentration of quercetin (in 20% extracts the quercetin concentration was of the magnitude of $10^{-5}M$ and myricetin $10^{-6}M$). The foregoing statements should not be construed as meaning that quercetin derivatives are the principal flavones of all plants. Although quercetin is the most common of the flavones (Gripenberg, 1962) some plants are devoid of quercetin yet contain appreciable quantities of other flavones. In this laboratory, Cofer (1963) recently found that the principal flavone aglycone of turnip greens was apparently not quercetin. The aglycone has not as yet been identified.

Quantitative recovery and antioxidant activity of quercetin. The quercetin concentrations of the extracts were determined spectrophotometrically in absolute ethanol at 258 mμ on chromatographically isolated and purified quercetin. For this comparison the molar absorptivity (2.1×10^4) was deter-

Table 4. Characteristics of flavone aglycones.

Extract	R_f values from two-dimensional chromatography				λ max in ethanol	
	No. aglycones	First solvent BAW	Second solvent 15% acetic acid	Second solvent 80% phenol		
Green onion	1	0.63	0.04	0.32	258	369
Green onion tops	2	0.64	0.05	0.33	257	370
		0.44	0.12	0.15	254	373
Green pepper	1	0.63	0.06	0.31	257	368
Green pepper seed	1	0.62	0.05	0.32	260	369
Potato	0	.....	.....	.....	.....	.....
Potato peel	1	0.61	0.05	0.30	258	369
Quercetin		0.62	0.05	0.31	258	368
Myricetin		0.42	0.11	0.14	255	375

mined from standard quercetin solutions. The quantitative recovery of quercetin and its antioxidant activity for 20% extracts are shown in Table 5. The extracted quercetin

Table 5. Quercetin fraction from vegetables as antioxidants on roast beef slices.

Source	Quercetin concentration $\times 10^{-5}M$	TBA number (3 day at 3°C)
Control	.....	8.2
Potato peel	2.1	4.6
Green onion	2.4	4.1
Green pepper	2.6	3.8
Quercetin	5.0	2.9
Green onion top	6.3	2.5
Green pepper seed	6.8	2.2

represents a quercetin content in 100 g of the original plant tissue ranging from 2.7 mg for potato peels to 13.1 mg for green onion tops. Since the tissues were not extracted exhaustively, these figures were only approximations. From three to seven assays were made on different samples of each plant material; the variations in quercetin for any one plant source were within ±20% of the mean value.

The antioxidant activity of standard quercetin is compared to other standard flavone aglycones in Table 6. In the present study,

Table 6. Antioxidant activity of standard flavone aglycones.

Aglycone	Antioxidant index ($5 \times 10^{-4}M$)
Quercetin (3,5,7,3',4'-pentahydroxy)	3.5
Fisetin (3,7,3',4'-tetrahydroxy)	3.7
Myricetin (3.5,7.3',4',5'-hexahydroxy)	4.3
Robinetin (3,7,3',4',5'-pentahydroxy)	4.3
Rhamnetin (3,5,3',4'-tetrahydroxy 7-methoxy)	1.9

hydroxylation at both the 5 and 7 positions did not lower antioxidant activity. This is evidenced by the findings that quercetin and fisetin had relatively the same activity, and myricetin possessed the same activity as robinetin. The antioxidant activity of rhamnetin was considerably below that of quercetin, showing that the 7-hydroxyl group is desirable. Dihydroquercetin was found to have the same antioxidant activity as quercetin, indicating either that the 2,3 double bond is not of major importance to antioxidant activity or that conversion of dihydroquercetin to quercetin took place while the compound was in contact with the oxidizing fat. However, chromatographic tests demonstrated that dihydroquercetin was not converted to quercetin by the hydrolysis procedure. Mehta and Seshadri (1959) suggested that conversion might account for the antioxidant activity of dihydroquercetin.

An additional hydroxyl group at the 5' position increased antioxidant activity over compounds having 3',4' hydroxylation only. Robinetin and myricetin had greater activity than quercetin and fisetin. No flavone aglycones were tested that possessed monohydroxylation of the B ring.

Since all flavones have the carbonyl group in the pyrone ring, no conclusions can be drawn from the data presented concerning the importance of this group. However, comparison of dihydroquercetin to D-catechin (the only structural difference being the carbonyl at the 4-position of dihydroquercetin) shows the importance of the carbonyl in flavonoids without the 2,3 double bond. The respective antioxidant indices for dihydroquercetin and catechin are 3.7 and 1.4.

Summarizing the relations between structure and antioxidant activity from the limited number of comparisons available in this study, it appears that, in the artificial aqueous lard system used: 1) dihydroxylation of the B ring increases antioxidant activity, 2) unsaturation of the pyrone ring is not essential for activity, 3) 5,7 hydroxylation of the chromone system did not decrease activity, and 4) 3-glycosylation of 3-hydroxylflavone does not alter its activity. More work is needed to determine the influence of glycosylation at other positions in the flavone structure.

REFERENCES

Bate-Smith, E. C. 1948. Paper chromatography of anthocyanins and related substances in petal extracts. *Nature* **161**, 835.

Bate-Smith, E. C. 1954. Flavonoid compounds in foods. *Advances in Food Research* **5**, 261.

Bate-Smith, E. C. 1959. "Pharmacology of Plant Phenols." (J. W. Fairbairn, ed.) Academic Press.

Cofer, A. 1963. Unpublished data. Florida State University.

Crawford, D. L., R. O. Sinnhuber, and H. Aft. 1961. The effects of methylation upon the antioxidant and chelating capacity of quercetin and dihydroquercetin in a lard substrate. *J. Food Sci.* **26**, 139.

Gripenberg, J. 1962. Flavones. Chapter 13 in "The Chemistry of Flavonoid Compounds." (T. A. Geissman, ed.) Macmillan Company, New York.

Harborne, J. B. 1959. The chromatography of the flavonoid pigments. *J. Chromatography* **2**, 581.

Heimann, W., and F. Reiff. 1953. The relation between chemical constitution and antioxidant activity of flavonols. *Fette u. Seifen* **55**, 451.

Heimann, W., A. Heiman, M. Gremminger, and H. H. Holman. 1953. Quercetin as an antioxidant. *Fette u. Seifen* **55**, 394.

Hillis, W. E., and T. Swain. 1959. The phenolic constituents of Prunus domestica. II. The analysis of tissues of the Victoria plum tree. *J. Sci. Food Agr.* **10**, 135.

Jurd, L., T. A. Geissmann, and M. K. Seikel. 1957. The flavonoid constituents of Spirodela oligorrhiza. II. The flavone constituents. *Arch. Biochem. Biophys.* **67**, 284.

Kefford, J. F. 1959. The chemical constituents of citrus fruits. *Advances in Food Research* **9**, 286.

Kelley, G. G., and B. M. Watts. 1957. Effect of copper chelating agents on the pro-oxidative activity of ascorbic acid with unsaturated fats. *Food Research* **22**, 308.

Kurth, E. F., and F. L. Chan. 1951. Dihydroquercetin as an antioxidant. *J. Am. Oil Chemists' Soc.* **28**, 433.

Lea, C. H. 1958. Some nutritional and allied problems confronting the food manufacturer: Technological aspects of antioxidants. *J. Sci. Food Agr.* **9**, 621.

Lea, C. H. 1962. The oxidative deterioration of food lipids. Chapter 1 in "Symposium on Foods: Lipids and Their Oxidation." (H. W. Schultz, ed.) Avi Publishing Co.

Lea, C. H., and P. A. T. Swoboda. 1956. On the antioxidant activity of the flavonols, gossypetin and quercetagetin. *Chem. & Ind.* 1956.

Lewis, E. J., and B. M. Watts. 1958. Antioxidant and copper binding properties of onions. *Food Research* **23**, 274.

Mehta, A. C., and T. R. Seshadri. 1959. Flavonoids as antioxidants. *J. Sci. Ind. Research* **18B**, 24.

Ramsey, M. B. 1962. Unpublished thesis. Florida State University.

Richardson, G. A., M. S. El-Rafey, and M. L. Long. 1947. Flavones and flavone derivatives as antioxidants. *J. Dairy Sci.* **30**, 397.

Scarborough, D. A., and B. M. Watts. 1949. The prooxidant effect of ascorbic acid and cysteine in aqueous fat systems. *Food Technol.* **3**, 152.

Seikel, M. K. 1962. Chromatographic method of separation, isolation and identification of flavonoid compounds. Chapter 3 in "The Chemistry of Flavonoid Compounds." (T. A. Geissman, ed.) Macmillan Company.

Simpson, T. H., and N. Uri. 1956. Hydroxyflavones as inhibitors of anaerobic oxidation of unsaturated fatty acids. *Chem. & Ind.* 1956.

Swain, T. 1962. Economic importance of flavonoid compounds: Foodstuff. Chapter 16 in "The Chemistry of Flavonoid Compounds." (T. A. Geissman, ed.) Macmillan Company.

Tarladgis, B. G., B. M. Watts, M. T. Younathan, and L. Dugan, Jr. 1960. A distillation method for the quantitative determination of malonaldehyde in rancid food. *J. Am. Oil Chemists' Soc.* **37**, 44.

Wu, M., and R. C. Burrell. 1958. Flavonoid pigments of the tomato (Lycopersicum esculentum Mill.) *Arch Biochem. Biophys.* **74**, 114.

Seasonal Changes Occurring in the Pectinesterase Activity and Pectic Constituents of the Component Parts of Citrus Fruits II. Pineapple Oranges[a]

A. H. ROUSE
University of Florida Citrus Experiment Station
AND
C. D. ATKINS AND E. L. MOORE
Florida Citrus Commission, Lake Alfred, Florida

(Manuscript received June 24, 1963)

SUMMARY

Pectinesterase activity, 3 pectic fractions, and other characteristics were determined periodically on 5 component parts of Pineapple oranges during a 9-month maturation cycle for 2 seasons. Generally, PE activity was greatest in the peel, membrane, and juice sacs in March, April, and May, when the Brix/acid ratio was highest. However, the activity varied in similar components for like months during the 2 seasons. The order of component parts for PE in most cases, from highest to lowest activity, was juice sacs, membrane, peel, seeds, and juice. Total PE in the average whole orange attained maximum activity in March and April. Over 52% of the activity present was found in the juice sacs, which represented about 22.5% of the whole fruit. Water-soluble pectin increased slightly in the peel and membrane, remained somewhat irregular in the juice sacs, and decreased to a constant level in the seeds throughout the growing season. Ammonium-oxalate-soluble pectin in the peel decreased slightly, and in the other components was either irregular or increased slightly, during maturation. Quantity of protopectin was greatest in the membrane and generally decreased toward the end of the sampling period in the various components. Total pectin and weight of the average whole orange was greater in the 1961–62 season than in the preceding season.

INTRODUCTION

The authors (Rouse *et al.*, 1962) recently reported on the pectinesterase activity (PE) and pectic content occurring in the component parts of the Valencia orange during maturation. The present paper is concerned with the enzymic activity and pectic content in the various component parts during growth and development of the Pineapple orange. It is the most widely grown mid-season orange in Florida, and, because of the excellent flavor and color of the juice (second only to the Valencia orange), approximately 35 million boxes were processed into frozen orange concentrate and single-strength juice during the 1961–62 season.

Research on seasonal changes in the pectic substances has placed emphasis upon the Valencia orange. Gaddum (1934), Sinclair and Jolliffe (1961), and Rouse *et al.* (1962) have reported on the pectic changes in the various components of the Valencia orange at different stages of maturity. Rouse (1953) reported the distribution of PE and total pectin in 6 component parts of matured Pineapple orange, and that juice sacs contained the most PE units, while the greatest amount of pectin was found in the membrane.

This investigation was made to determine variations due to seasonal changes in PE activity and in the 3 pectic fractions of component parts of Florida Pineapple oranges. These pectic fractions are water-soluble, ammonium-oxalate-soluble (the insoluble pectates and pectinates), and sodium-hy-

[a] Cooperative research by the Florida Citrus Experiment Station and the Florida Citrus Commission. Florida Agricultural Experiment Stations Journal Series, No. 1682.

droxide-soluble or protopectin. Changes in weight, total solids of the whole orange, percentage by weight of the component parts, and soluble solids/acid ratio of the juices were also determined during maturation of the fruit. The component parts of the fruit consisted of peel, membrane, juice sacs, seeds, and juice. Data are also presented on the PE activity and total pectin present in the whole orange during the growth cycle.

EXPERIMENTAL PROCEDURE

Preparation of samples. Pineapple oranges grown on rough lemon rootstock were picked the first of each month (September through May) for 2 years from the same trees, washed, dried, and separated by hand into peel, membrane, juice sacs, seeds, and juice. These components are briefly described as follows:

1) Peel consists of the flavedo, or epicarp, which is the outer colored portion of the peel, and the albedo, or the white spongy portion, known as the inner mesocarp. This also includes the central core.
2) Membrane is the segment membrane, or endocarp tissue, enclosing the juice sacs. This is sometimes referred to as the rag.
3) Juice sacs are the vesicles that hold the juice.
4) Seeds.
5) Juice is the liquid expressed from the sacs.

From 15 to 24 oranges, depending on fruit size, were used in preparing the samples of component parts. The peel, membrane, and seeds were individually comminuted in an Osterizer, whereas the juice was expressed from the juice sacs by a Watson-Stillman hydraulic press to 40 psig.

Procedures in preparing the component parts for PE determination and for alcohol-insoluble solids (AIS) were as described by Rouse *et al.* (1962) for Valencia orange. Pectic fractions were extracted from the AIS of the various components.

Methods of analysis. PE activity and the 3 pectic fractions were determined and reported on a dry-weight basis as described by Rouse *et al.* (1962).

Soluble solids as degrees Brix by spindle at 17.5°C, total acid as citric, and moisture contents were determined by conventional methods. Ratios of total soluble solids to acid were calculated. Obtained by calculation were total solids, PE activity, and total pectin of the average whole orange.

RESULTS AND DISCUSSION

Eleven healthy Pineapple orange trees were selected from Block II at the Citrus Experiment Station. No appreciable difference was noted in growth, production, or fruit quality of these trees during this study. Seasonal differences were found in some characteristics of Pineapple oranges during the two growth seasons, as were found for the Valencia oranges (Rouse *et al.*, 1962).

Table 1 presents some general characteristics of Pineapple oranges for a 9-month period during the 1960–61 and 1961–62 seasons. The weights of the average oranges in the two seasons were, respectively, 128 and 165 g in September, and 181 and 245 g in May. Also, for corresponding months during the two seasons, the average total solids of the whole oranges were from 15.7 and 16.1% to 18.1 and 18.8%. Total soluble solids in the juices were respectively 8.55–12.96° and 9.38–14.10° Brix in 1960–61 and 1961–62. The Brix/acid ratios of the extracted juices were slightly higher for the 1960–61 season, with slightly lower soluble

Table 1. Comparison of Pineapple orange characteristics in relation to maturity during 2 citrus seasons.

Sampling time	1960-61				1961-62			
	Orange average wt. (g)	Total solids of av. whole orange (%)	° Brix of juice	Brix/acid ratio of juice	Orange average wt. (g)	Total solids of av. whole orange (%)	° Brix of juice	Brix/acid ratio of juice
September	128	15.7	8.55	5.12	165	16.1	9.38	4.62
October	162	15.0	8.65	7.21	196	17.0	10.05	6.57
November	164	16.2	9.90	9.71	175	18.1	11.40	9.19
December	158	17.6	11.70	12.58	199	18.2	12.10	10.80
January	172	18.1	12.00	13.64	201	18.1	12.85	12.36
February	170	18.1	12.28	15.35	233	18.7	12.90	13.87
March	174	18.4	12.80	17.30	227	18.2	13.15	15.84
April	177	18.0	13.15	19.63	222	18.5	13.90	17.38
May	181	18.1	12.96	22.34	245	18.8	14.10	20.14

solids than those found for juices during the 1961–62 season.

Component parts of fruit. The percentages of component parts in Pineapple oranges examined during this 2-year study are presented in Table 2 for each month, September through May. Actually, the percentage of an individual component did not vary greatly during this part of the maturation period, except for the seeds, which generally decreased as the fruit matured and became larger. In 1960–61 the quantity of extracted juice was greatest (44.2%) in oranges harvested in November, whereas in 1961–62 the juice yield was greatest (approx. 40.0%) in November, December, and March. The order of least to greatest quantity of component parts was seeds, membrane, peel, juice sacs, and juice. However, in 1961–62 the percentage of juice sacs was less than that of the peel. This was due to the sudden increase in peel and decrease in juice sacs during the last 3 months of maturation. The respective average percentage values for each of these components were 3.8, 13.7, 19.9, 23.6, and 39.0 in 1960–61, and 3.5, 14.2, 22.7, 21.9, and 37.7 in 1961–62. There was slightly less peel and more juice sacs and juice in the average whole orange the first year, and practically no difference between the 2 citrus seasons in membrane and seeds.

Pectinesterase activity. Table 3 shows the distribution of PE activity, on a dry-weight basis, in the component parts of Pineapple oranges for 2 seasons. Peel, membrane, and juice sacs were the most active in PE, and in most instances these 3 components were more active in PE the last 3 months of this maturation study, when the Brix/acid ratios were greatest.

The order of component parts for PE, from lowest to highest activity, was juice, seeds, peel, membrane, and juice sacs. This

Table 2. Distribution of the component parts in Pineapple oranges during 2 citrus seasons.

Sampling time	1960-61					1961-62				
	Peel (%)	Membrane (%)	Juice sacs (%)	Seeds (%)	Juice (%)	Peel (%)	Membrane (%)	Juice sacs (%)	Seeds (%)	Juice (%)
September	24.9	13.2	20.1	5.3	36.5	25.6	14.7	18.9	4.7	36.1
October	19.1	12.7	28.8	4.0	35.4	21.4	14.1	22.8	4.8	36.9
November	17.3	13.1	21.6	3.8	44.2	20.4	14.0	22.2	3.6	39.8
December	16.0	14.1	24.5	3.5	41.9	21.4	14.4	20.5	3.9	39.8
January	18.9	15.3	21.6	3.7	40.5	20.1	12.8	28.8	3.2	35.1
February	20.1	13.9	24.5	3.3	38.2	23.4	13.8	25.8	2.8	34.2
March	19.8	13.6	22.5	3.9	40.2	22.8	15.8	18.2	3.1	40.1
April	21.8	12.4	24.6	3.2	38.0	24.0	14.5	19.7	3.0	38.8
May	21.1	15.0	23.9	3.3	36.7	25.4	13.9	19.7	2.2	38.8
Av.	19.9	13.7	23.6	3.8	39.0	22.7	14.2	21.9	3.5	37.7

Table 3. Distribution of pectinesterase activity in the component parts of Pineapple oranges during 2 citrus seasons.

Sampling time	1960-61 (PE.u.)g total solids × 1000					1961-62 (PE.u.)g total solids × 1000				
	Peel	Membrane	Juice sacs	Seeds	Juice	Peel	Membrane	Juice sacs	Seeds	Juice
September	45.5	52.2	221.4	7.5	2.6	69.9	54.5	341.5	9.8	1.2
October	61.8	51.5	300.9	5.5	2.6	61.0	57.1	296.7	4.1	3.3
November	73.8	147.6	313.9	5.2	1.1	68.9	79.0	272.5	3.8	1.9
December	50.8	59.5	298.4	2.2	0.3	72.0	120.1	367.6	5.1	2.6
January	54.9	112.8	337.5	4.0	0.9	63.8	80.7	281.1	5.1	2.5
February	51.1	79.7	286.4	3.5	0.9	51.0	85.4	251.5	2.5	1.6
March	141.3	198.4	597.4	3.0	0.8	83.5	129.5	349.0	6.6	3.2
April	143.2	184.5	479.8	4.1	1.6	72.5	122.6	362.8	4.9	2.2
May	84.3	158.0	408.3	3.6	0.8	64.7	123.6	294.4	4.7	0.7
Av.	78.5	116.0	360.4	4.3	1.3	67.5	94.7	313.0	5.2	2.1

Table 4. Total pectinesterase activity and total pectin in the average Pineapple whole orange during 2 citrus seasons.

Sampling time	1960-61			1961-62		
	Total PE × 10^3 in fruit	PE due to juice sacs (%)	Total pectin in fruit (g)	Total PE × 10^3 in fruit	PE due to juice sacs (%)	Total pectin in fruit (g)
September	1,303	56.8	2.56	2,362	57.5	3.29
October	2,261	67.1	3.27	2,695	60.0	3.01
November	2,510	60.1	2.53	2,686	56.9	3.79
December	2,277	69.7	2.27	3,824	58.2	3.34
January	3,014	63.0	2.45	3,508	66.3	3.89
February	2,614	65.7	2.46	3,565	61.9	4.98
March	5,830	60.5	2.28	4,259	52.0	4.17
April	5,203	55.9	2.38	4,368	57.8	3.97
May	4,292	59.8	2.79	4,261	56.1	3.93

order was the same as found in Valencia oranges by Rouse *et al.* (1962). The respective average PE values for these components were 1.3, 4.3, 78.5, 116.0, and 360.4 in 1960–61, and 2.1, 5.2, 67.5, 94.7, and 313.0 in 1961–62. The highest PE value reported by Rouse (1953) in mature Pineapple oranges was 794.5 units for juice sacs, whereas the highest value for this component was found to be 597.4 units during this 2-season study.

Total PE activity for the average whole orange is shown in Table 4 for each month of the maturation study. Over 52% of the enzymic activity came from the juice sacs, which represented approximately 22.5% of the whole orange for the 2 seasons.

Pectic substances. Individual values of the water-soluble, ammonium-oxalate-soluble, sodium-hydroxide soluble, and total pectins for each component part for each month during 2 seasons, were averaged together (Figs. 1–4). Because of the low concentration of pectic fractions in the juices, their average amounts are not shown. Total pectin ranged from 0.37 to 0.60%, of which the water-soluble pectins corresponded to 74–84%.

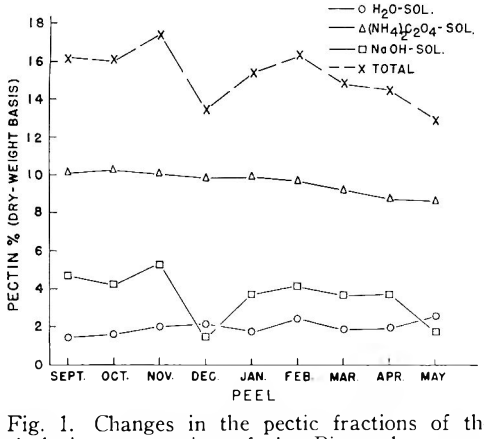


Fig. 1. Changes in the pectic fractions of the peel during maturation of the Pineapple orange. Each curve represents the mean values for 1960–61 and 1961–62 seasons.

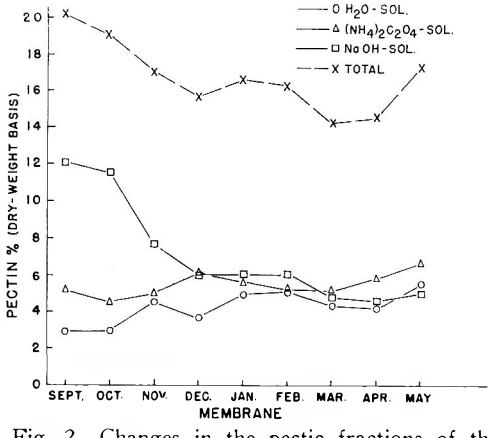


Fig. 2. Changes in the pectic fractions of the membrane during maturation of the Pineapple orange. Each curve represents the mean values for 1960–61 and 1961–62 seasons.

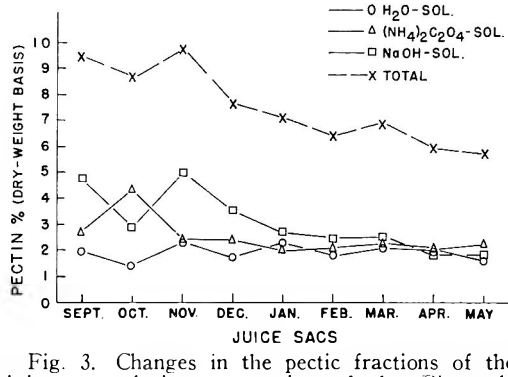


Fig. 3. Changes in the pectic fractions of the juice sacs during maturation of the Pineapple orange. Each curve represents the mean values for 1960–61 and 1961–62 seasons.

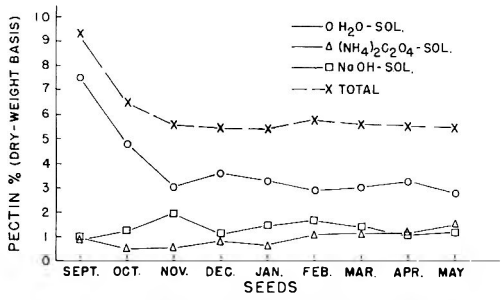


Fig. 4. Changes in the pectic fractions of the seeds during maturation of the Pineapple orange. Each curve represents the mean values for 1960–61 and 1961–62 seasons.

Total pectin for the average whole orange is shown in Table 4 for each month of the maturation study. The quantity of total pectin per average orange was greater in 1961–62 than in 1960–61, but the fruit was also heavier (Table 1) during the second year. However, the range of percent total pectin was similar in the whole fruit, being respectively 1.4–2.0% and 1.5–2.1%, for 1960–61 and 1961–62.

Peel. Fig. 1 shows changes in pectic fractions on a dry-weight basis for the peel of Pineapple oranges. Water-soluble pectin generally increased slightly throughout the growth cycle. The quantity of ammonium-oxalate-soluble pectin was greatest in this component, varying from 10.23 to 8.69% and decreasing slowly with maturity. Sodium-hydroxide-soluble pectin, protopectin, increased to a high of 5.36% in November, and decreased to its lowest (1.39%) in December, after which it increased and leveled off for several months and then, in May, dropped suddenly to 1.72%. Total pectin, of course, resulted from the sum of the 3 pectic fractions, the highest (17.46%) in November and the lowest (13.03%) in May.

Membrane. Fig. 2 shows the percentages of the pectic fractions in membrane. Total pectin is most abundant in the membrane because of its excellent source of water-soluble pectin, and particularly of the sodium-hydroxide-soluble pectin present. Trends in the curves for both water- and ammonium-oxalate-soluble pectins were to increase generally throughout the maturation season. Water-soluble pectin varied from 2.90%, in September, to 5.57%, in May. In September and October, the immature fruit contained 12.06 and 11.57%, respectively, of the sodium-hydroxide-soluble fraction, which decreased to 7.63% in November and continued to diminish during maturity to 4.58% in April. Total pectin (20.18%) was greatest in September and least (14.23%) in March.

Juice sacs. This component is of more concern to the citrus processor of frozen concentrated orange juice because it contains a high potential of PE activity in the tissue, and a large portion of the pulp in citrus juice consists of juice sacs. Because of the importance to processors and variation of PE in this component, PE activities were plotted for each season (Fig. 5). In

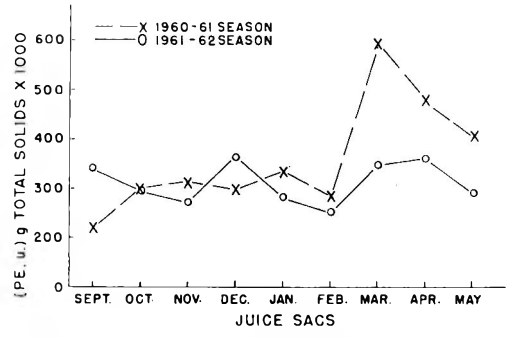


Fig. 5. Changes in the PE activity of the juice sacs during maturation of the Pineapple orange for the 1960–61 and 1961–62 seasons.

1960–61 an activity of 597.4 units was obtained in March, whereas in 1961–62 two peaks of activity were reached, one in December and the other in April, respectively 367.6 and 362.8 units.

All 3-pectic fractions (Fig. 3) were very irregular during the first 3 months of the maturation cycle. Generally, the water- and ammonium-oxalate-soluble pectins varied very little from December to May, whereas the sodium-hydroxide-soluble fraction decreased from a high (5.01%) in November to a low (1.89%) in May. Total pectin in the juice sacs is the primary source of pectin in citrus juices, varying from 9.82 to 5.77%.

Seeds. Seeds account for approximately 3.6% (Table 2) of the average whole Pineapple orange, being somewhat greater when the orange is immature and generally less as the fruit becomes larger (Table 1). Total solids of the seeds varied from 26.6% to

53.4%, the lower percentage when the Brix/acid ratio was low, and the higher percentage when this ratio was greater. This coincided with that found for Valencia orange seeds (Rouse *et al.,* 1962). Water-soluble pectin (Fig. 4) definitely decreased from 7.50% in September to 3.05% in November, and continued to be almost constant for the remainder of the growing cycle. General patterns for the oxalate- and sodium-hydroxide-soluble fractions were low, the former ranging from 0.97 to 1.50% and the latter from 0.86 to 1.97%. In most cases, total pectin was influenced by the large quantity of water-soluble pectin present.

REFERENCES

Gaddum, L. W. 1934. The pectic constituents of citrus fruits. Florida Univ. Agr. Expt. Sta. Bull. No. 268, p. 9.

Rouse, A. H. 1953. Distribution of pectinesterase and total pectin in component parts of citrus fruits. *Food Technol.* **7**, 360.

Rouse, A. H., C. D. Atkins, and E. L. Moore. 1962. Seasonal changes occurring in the pectinesterase activity and pectic constituents of the component parts of citrus fruits. I. Valencia oranges. *J. Food Sci.* **27**, 419.

Sinclair, W. B., and V. A. Jolliffe. 1961. Pectic substances of Valencia oranges at different stages of maturity. *J. Food Sci.* **26**, 125.

Effect of Ionizing Radiations on Plant Tissues. III. Softening and Changes in Pectins and Cellulose of Apples, Carrots, and Beets[a]

Z. I. KERTESZ,[b] R. E. GLEGG,[c] F. P. BOYLE,[d]
G. F. PARSONS, AND L. M. MASSEY, JR.
Department of Food Science and Technology, New York State Agricultural Experiment Station, Cornell University, Geneva, New York

(Manuscript received June 24, 1963)

SUMMARY

A study was conducted of changes that occur in pectic and cellulose constituents of apple, carrot, and beet tissues as a result of treatment with gamma radiation. In apple tissues, degradation of both these constituents occurred at approximately the dose at which tissue softening could be first demonstrated, and progressed with increasing dose. The degradation of pectins was demonstrated by several criteria, such as the loss of viscosity in the individual fractions, the loss of specific viscosity calculated for a constant uronide or calcium pectate value, and by change in the soluble-insoluble pectin ratio. Cellulose degradation was characterized by a decrease in the specific viscosity calculated for 0.15% cellulose solutions.

Although these findings were corroborated in carrot tissues, the softening of beets does not seem to be as clearly accompanied by such marked changes in pectins and cellulose. Whereas previously published data indicated that beets have a higher softening threshold dose than either carrots or apples, this does not explain the differences found. It is suspected that other factors, such as cell turgidity, are involved in the softening process, and is here responsible for this phenomenon.

There are many observations in the literature that, when plant tissues are exposed to ionizing radiations exceeding approximately 25 krads, changes take place in the texture or firmness (Brasch and Huber, 1947; Morgan, 1955; Hannan, 1955). The threshold dose (TD) at which this change occurs differs significantly for various plant tissues (Glegg *et al.*, 1956; Boyle *et al.*, 1957). It is possible that such softening may be desirable in a few instances, but in most cases it is regarded as an undesirable side-effect.

The relation between the softening of plant tissues by ripening and exposure to heat and concurrent changes in the various pectic constituents is well established for many fruits and vegetables (Kertesz, 1951). However, only a few articles deal with the relationship of radiation-induced texture changes to *in vivo* alterations of pectins and none of cellulose. Roberts and Proctor (1955), using microchemical methods, found that irradiating potatoes with 2–3 mrads of cathode rays produced noticeable alterations in the middle lamellae and that these changes were accompanied by a soften-

[a] Approved by the Director of the New York State Agricultural Experiment Station, Geneva, New York as Journal Paper No. 1351, May 28, 1963. This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, QM Research and Engineering Command, U. S. Army, and has been approved for publication. The views or conclusions are those of the authors, and not to be construed as necessarily reflecting the views or endorsement of the Department of Defense.

[b] Present address: Nutrition Division, Food and Agriculture Organization of the United Nations, Rome, Italy.

[c] Present address: Eastman Kodak Company, Research Laboratory, Rochester, New York.

[d] Present address: 4316 Koae Place, Honolulu, Hawaii.

ing of the potatoes. Those authors did not measure the texture changes and the pectic alteration quantitatively. McArdle and Nehemias (1956) reported texture measurements on apples and carrots irradiated with gamma rays up to 2.5 mrads and found that the softening was accompanied by decrease in both protopectin and total pectins, whereas the proportion of soluble pectins and pectates increased. They state that the lowered viscosities of the various extracts indicated a "depolymerization of the pectin, pectate, and protopectin." Previous communications from this laboratory have also presented evidence for the depolymerization of both pectin (Skinner and Kertesz, 1960; Kertesz *et al.*, 1956) and cellulose (Glegg and Kertesz, 1957), but in model *in vitro* systems.

Of the various fruits and vegetables that might be likely prospects to exhibit certain benefits from radiation treatment, apples, carrots, and beets appeared to possess texture properties that might be critical to product acceptability, and at the same time were readily adaptable to the types of measurements and analysis planned in this study. We have previously reported some objective measurements of the radiation-induced firmness changes in several varieties of these tissues (Glegg *et al.*, 1956; Boyle *et al.*, 1957). The present paper deals with concomitant changes in the pectic constituents and cellulose of these samples. Although our primary interest involves the early stage of radiation-induced tissue softening rather than the advanced stages, where the tissue characteristics have been extensively changed, data were obtained on a wide range of firmness.

METHODS AND MATERIALS

The apples used were of the varieties McIntosh, Rome Beauty, and Baldwin. The fruit was grown in the Experiment Station orchards, picked at a "commercial" state of maturity, and stored for a maximum of 3 months at 31°F until use. Carrots of the variety Red Core Chantenay were harvested from Experiment Station plots in early November, and held in common storage for approximately 2 months before use. The beets, of the variety Detroit Dark Red, were obtained from local sources and held in common storage for less than 2 months before use. All fruits and vegetables were in excellent physiological condition at time of use. Because of the physical limitations of the radiation source used, it was not possible to irradiate intact samples in numbers sufficient for statistical significance or for dose uniformity. Hence, pieces of tissue were used in the form of cylinders 18 mm in diameter and 10 mm in height, wrapped in Saran film and sealed with cellophane tape. This packaging almost completely prevented the browning of apple tissue during the duration of the experiment and also averted any serious change in moisture content. No other physiological changes were observed. Although atmospheric composition in and around the tissue was not measured, almost certainly conditions of high carbon dioxide and low oxygen were built up under the 22–26-hour period of handling at ambient temperatures between 21 and 25°C. Other experiments have indicated that no significant change in composition of the pectin and cellulose content of these tissues may be attributed directly to the specific environmental conditions experienced here. Further, other experiments conducted with intact apple tissues have verified the validity of the changes observed in more detail in these experiments. The possibility of artifacts due to the experimental protocol must be borne in mind, however. Other details of these samples have been previously reported (Boyle *et al.*, 1957).

Irradiation. Irradiation with Cobalt-60 gamma rays was performed in the same manner as previously described in another report of this series (Glegg *et al.*, 1956). Briefly, irradiation was conducted at various dose rates established by varying the distance from the source over an 18-hour exposure period. This was accomplished by placing the tissue pieces in horizontal planes directly over the source, at locations previously determined to afford radiation-field uniformity to within ±5%. Within 4–8 hours of irradiation, the firmness of the tissue cylinders was measured in resistance to crushing load in a device described previously (Glegg *et al.*, 1956; Moyer *et al.*, in preparation).

Preservation of tissue samples. Immediately after firmness measurement, the tissue cylinder remnants were weighed and put into a twofold weight of boiling 95% ethanol. The ethanol was brought back to a boil, and boiling was continued for 3 min. After cooling and standing overnight at room temperature, the samples were ground in a Waring blender and again allowed to stand overnight at room temperature. Following a series of filtrations through nylon cloth and replacement with 70%, then with 95%, and finally with absolute ethanol, the tissue samples were allowed to drain. Further drying was accomplished first in air and then in vacuum over P_2O_5. These samples

of alcohol-insoluble solids (AIS) were stored for subsequent determinations of pectin fractions and cellulose.

Pectin extraction and evaluation. The pectin extractions were accomplished by a modification of methods described in detail elsewhere (Kertesz, 1951). Briefly, the extractions were made through fritted-glass filters under constant temperature conditions as described by Weihe and Phillips (1947). Samples of AIS weighing 0.25 g were extracted with three solvents. The first three extractions were each made with 15 ml of water, at 30°C. Then the residue was extracted 3 times with a solution containing 0.2% Calgon and 0.8% NaCl at 30°C, and finally three times more with 0.05*N* HCl at 80°C. Then the residue was rinsed with 3 ml of water, and this was added to the acid extract. The water, Calgon and acid extracts were collected separately and made up to 50 ml each with enough Calgon and NaCl added to give respective final proportions of 0.2 and 0.8%. The extracts were all adjusted to pH 6.0±0.2. Following viscosity measurement, the residues were kept in aqueous suspension for subsequent use in cellulose determination.

Viscosities of the three separate fractions were determined at 30°C in Ostwald-Cannon-Fenske pipettes. In many cases the viscosities were also determined in a series of dilutions, using a solution containing a 0.2% Calgon and 0.8% NaCl as diluent. The "average specific viscosity" values were obtained by averaging the results obtained on the water, Calgon, and acid extracts. Uronic acids were determined by the McComb and McCready (1952) method.

Cellulose extraction and evaluation. Each of the residues from the pectin determinations was digested overnight with 1% (filtered) Pectinol 100D solution, and washed four times with hot water, twice with 95% ethanol, twice with absolute ethanol, and finally with ether, using about 25 ml liquid each time and centrifuging after each washing. The residue was then dried in vacuum over P_2O_5 and weighed.

For the determination of cellulose solution viscosity, 12.5 ml of water was added to the residue in a 25-ml Erlenmeyer flask and allowed to stand overnight. Residue was then broken up into fine pieces with a stirring rod, and 2.5 ml of cuprietylenediamine (CED) were added. The flask was next flushed out with nitrogen, and the sample was swirled for 10 min. Then 10 ml more of CED were added, the flask was again flushed with nitrogen, and the sample was shaken for 15 min. The solution was transferred to a centrifuge tube with glass stopper, flushed again with nitrogen, and centrifuged for 15 min at 2,000 rpm. At least 16 ml of the supernatant was poured off to be used for viscosity determinations. Viscosity was determined on the original solution, and on 3 further dilutions made with mixtures of equal volume of CED and water. The solution was recovered as completely as possible from pipettes, flasks, etc., plus the washings from the residue. The CED-insoluble matter (the insoluble residue) was washed into a weighed fritted-glass filter and washed once with 50% alcohol. Then it was dried 3 hr at 105°C, cooled for 0.5 hr, and weighed.

The cellulose was precipitated from the combined recovered CED solutions by the slow addition of 27 ml of 5*N* H_2SO_4. The mixture was centrifuged for 15 min at 2,000 rpm, and the supernatant was removed by filtration. The cellulose residue was resuspended, and washed 3 times with water and once with 50% alcohol. The precipitate was aspirated as dry as possible, and then oven-dried overnight at 105°C. The percentages of cellulose were then calculated as previously reported (Kertesz *et al.*, 1958).

RESULTS AND DISCUSSION

Alcohol-insoluble-solids (AIS) content of our samples (irradiated and unirradiated) ranged as follows: Apples, Baldwin 1.56–2.21%, Rome 1.53–1.74%, and McIntosh 1.73–2.03%; carrots, 2.20–2.42%; beets, 2.33–3.16%; apple purée, 3.01–3.80%; and beet purée, 2.57–2.86%. There was no significant correlation between AIS and radiation dose. Therefore it is concluded that irradiation within this dose range has no detectable effect on this constituent.

In respect to the pectin analysis with apples (the tissue studied most extensively) viscosity measurements (both independent of and corrected for a given concentration of uronide) have been emphasized. Increased doses of radiation caused a progressive increase in the viscosity of the water extracts. The Calgon extracts showed a slight viscosity increase at first, but above 100 krad showed a progressive decrease. The most pronounced and consistent change occurred in the acid-extracted fractions, which in all instances showed a definite progressive decrease in viscosity. For the sake of brevity, only the data for the McIntosh determinations are presented in Table 1. The data for other varieties followed all trends indicated.

The changes that occur in the pectins of all the irradiated apple tissues are shown graphically by plotting the average specific

Table 1. Observations on the pectic and cellulose constituents of irradiated McIntosh apple tissues.

Dose (krad)	Firmness (lb)	AIS (%)	Pectin								Cellulose			
					Calcium pectate			Uronide						
			Spec. visc.[a]		In AIS (%)	In tissue (%)	W[b]/CH	In AIS (%)	In tissue (%)	W[c]/CH	Spec. visc. (0.05% anhydrogalacturonic acid)	Intrinsic visc.	In tissue (%)	Spec. visc. (0.15% cellulose)
None	72	2.03	W	0.298	6.80	0.138	0.29	4.68	0.095	0.341	0.382	8.7	0.41	2.7
			C	0.179	5.52	0.112		2.62	0.053		0.409			
			H	0.680	18.1	0.367		11.1	0.225		0.364			
			Av.	0.386 Total	30.4	0.617		18.40	0.373					
100	56	1.92	W	0.488	.....	.....	....	7.12	0.137	0.624	0.412	8.1	0.25	2.0
			C	0.157	.....	.....		2.56	0.049		0.365			
			H	0.413	.....	.....		8.84	0.170		0.278			
			Av.	0.353 Total				18.52	0.356					
200	44	1.73	W	0.482	11.0	0.191	0.63	8.10	0.140	0.816	0.355	6.6	0.40	1.6
			C	0.125	5.52	0.096		3.09	0.054		0.243			
			H	0.315	12.1	0.209		6.83	0.118		0.277			
			Av.	0.307 Total	28.6	0.496		18.02	0.312					
530	26	1.96	W	0.519	.....	.....	.....	8.57	0.168	0.744	0.345	3.1	0.40	0.58
			C	0.112	.....	.....		3.68	0.072		0.183			
			H	0.283	.....	.....		7.82	0.153		0.216			
			Av.	0.305 Total				20.07	0.393					
2060	0	1.90	W	0.303	17.0	0.322	1.01	10.5	0.200	1.450	0.172	4.6	0.29	0.94
			C	0.036	4.24	0.081		1.84	0.035		0.116			
			H	0.133	12.5	0.237		5.42	0.103		0.146			
			Av.	0.157 Total	33.7	0.640		17.76	0.338					

[a] Fraction W = soluble in cold water; C = soluble in 0.2% Calgon + 0.8% sodium-chloride; and H = soluble in 0.05*N* HCl (80°C).

[b] $\frac{W}{CH} = \frac{\%\ W\ \text{(calcium pectate)}}{\%\ C + \%\ H\ \text{(calcium pectate)}}$

[c] $\frac{W}{CH} = \frac{\%\ W\ \text{(uronide)}}{\%\ C + \%\ H\ \text{(uronide)}}$

viscosities of isolated pectins (independent of pectin concentration) vs. log dose of gamma radiation applied (Fig. 1). A thresh-

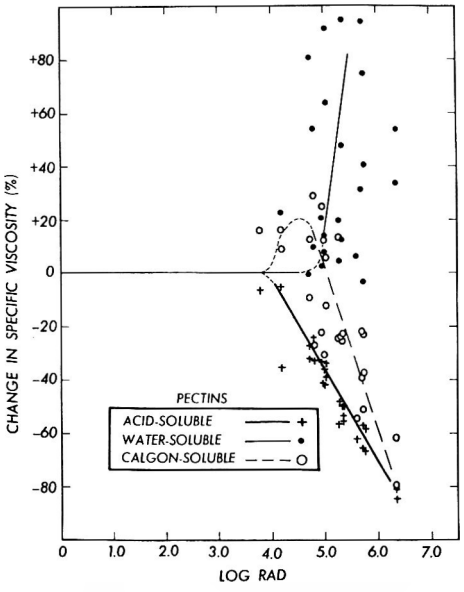


Fig. 1. Changes in the viscosities of three different pectin extracts from irradiated apple tissues.

old value for pectin changes of about 37 krad was obtained, which is very near to the 32 krad TD previously found for the softening of these tissues (Boyle *et al.*, 1957).

Further experiments on the concentration-viscosity relationship were conducted by determining uronide content and calcium pectate values. One series of such measurements is also presented in Table 1. The large change in the soluble vs. insoluble pectin ratio in both calcium pectate and uronide determinations substantiates the viscosity data and also indicates progressive solubilization of the originally Calgon- and acid-insoluble pectins.

The transient increase in viscosity of Calgon-soluble pectins noted at doses slightly above the threshold is of interest. Two possible explanations were considered. First, it seemed possible that the concentration of this fraction might have increased, resulting from reinforcement by products from partial degradation of the relatively more radiation-susceptible acid-soluble fraction. Second, it also seemed possible that an actual increase in the viscosity of this fraction might have resulted from radiation-induced cross-linking (Wahba *et al.*, 1963). To this point, we have found that the increase in viscosity of the Calgon-soluble fraction in the 10–100-krad range is accompanied by a corresponding increase in the anhydrogalacturonic acid content, an observation in harmony with the first of the above alternatives. The possibility of an actual increase in viscosity through radiation-induced cross-linking, although not specifically ruled out, is also considered relatively remote in view of the rather sharply restricted pH range through which this effect has been noted in model systems (pH 1.4–2.6).

Calcium pectate determinations were made on most, but not all, pectin extracts. For the apple samples, an average ratio of 0.569 for galacturonic acid anhydride vs. calcium pectate was obtained with a range of 0.434–0.734. This indicates that the calcium pectate values were almost twice as high for the pectin contents as those calculated from the uronic acid determinations. This is not entirely surprising in that calcium pectate values for purified pectins run about 110%, and that the calcium pectate precipitates were not purified or further analyzed for correction for non-pectic impurities. However, the ratio was constant enough to help in evaluation of the radiation-induced changes.

The total proportion of calcium pectate was found to be similar in the unirradiated and irradiated apples, an observation in harmony with the lack of changes in AIS content. The soluble-insoluble pectin fraction (calculated on the basis of the calcium pectate contents of the fresh tissues) increased irregularly with radiation dose. However, the degradation of the pectin in all three fractions brought about by increased irradiation dose is clearly demonstrated by both the direct viscosity measurements and the specific viscosity values expressed on the basis of 0.05% calcium pectate content.

Table 2 presents data obtained from the analysis of carrot tissue. Only two radiation doses were applied in these studies, both of which are above the softening threshold previously reported (approximately 170 krad). A progressive degradation of all pectin fractions with increasing radiation dose is indi-

Table 2. Observations of the pectic and cellulose constituents of irradiated Red Core Chantenay carrot tissues.

Dose (krad)	Firmness (lb)	Pectin: Calcium pectate: Spec. visc.[a]			In AIS (%)	In tissue (%)	W[b]/CH (tissue)	Spec. visc. (0.05% calcium pectate)	Cellulose: Intrinsic visc.	In tissue (%)	Spec. visc. (0.15%)
None	94	W	0.265		15.6	0.356	0.68	0.174	6.10	0.365	0.358
		C	0.580		14.8	0.337		0.394			
		H	0.085		8.28	0.189		0.103			
		Av.	0.310	Total	38.7	0.882					
500	70	W	0.210		15.7	0.380	0.66	0.133	5.00	0.426	0.265
		C	0.552		17.4	0.421		0.292			
		H	0.067		6.60	0.160		0.103			
		Av.	0.276	Total	39.7	0.961					
1900	32	W	0.151		16.6	0.361	0.70	0.091	4.70	0.447	0.258
		C	0.351		18.4	0.403		0.191			
		H	0.042		5.48	0.120		0.096			
		Av.	0.181	Total	40.5	0.887					

[a] Fraction W = soluble in cold water; C = soluble in 0.2% Calgon + 0.8% sodium chloride; and H = soluble in 0.05*N* HCl (80°C).

[b] $\frac{W}{CH} = \frac{\%\ W\ (\text{calcium pectate})}{\%\ C + \%\ H\ (\text{calcium pectate})}$

Table 3. Observations on the pectic and cellulose constituents of irradiated beet tissues.

Dose (krad)	Av. crushing load (lb)	Pectin: Calcium pectate: Spec. visc.[a]		In AIS (%)	In tissue (%)	W[b]/CH (tissue)	Spec. visc. (0.05% calcium pectate)	Cellulose: Intrinsic visc.	In tissue (%)	Spec. visc. (0.15%)
0	93.0	W 0.070		14.9	0.470	0.564	0.047	6.59	0.66	0.745
		C 0.004		3.88	0.123		0.011			
		H 0.100		22.5	0.712		0.044			
		Av. 0.058	Total	41.3	1.31					
60	.....	W 0.050		8.68	0.227	0.438	0.057	4.31	0.68	0.545
		C 0.004		5.60	0.146		0.007			
		H 0.079		14.2	0.371		0.056			
		Av. 0.044	Total	28.5	0.744					
125	96.1	W 0.052		6.96	0.218	0.535	0.037	5.84	0.65	0.700
		C 0.007		1.64	0.051		0.004			
		H 0.088		11.3	0.356		0.048			
		Av. 0.049	Total	19.9	0.625					
250	93.7	W 0.052		11.9	0.279	0.554	0.042	.....	.....	.....
		C 0.002		2.16	0.050		0.010			
		H 0.080		19.4	0.453		0.041			
		Av. 0.045	Total	33.6	0.782					
500	89.2	W 0.043		9.56	0.227	0.460	0.045	.....	.....	.....
		C 0.005		3.08	0.073		0.016			
		H 0.076		17.7	0.420		0.043			
		Av. 0.041	Total	30.4	0.720					
1000	84.4	W 0.049		6.52	0.199	0.444	0.044	4.78	0.60	0.555
		C 0.011		1.80	0.055		0.012			
		H 0.083		12.9	0.393		0.041			
		Av. 0.048	Total	21.2	0.647					
2000	71.0	W 0.041		9.08	0.253	0.563	0.039	4.59	0.54	0.500
		C 0.011		2.52	0.070		0.027			
		H 0.060		13.6	0.379		0.046			
		Av. 0.037	Total	25.2	0.702					

[a] Fraction W = soluble in cold water; C = soluble in 0.2% Calgon + 0.8% sodium-chloride; and H = soluble in 0.05N HCl (80°C).

[b] $\frac{W}{CH} = \frac{\%\ W\ \text{(calcium pectate)}}{\%\ C + \%\ H\ \text{(calcium pectate)}}$

cated. The doses are not extensive enough to indicate the possible occurrence of the transient increase in viscosity of the Calgon-soluble pectins at doses slightly above the threshold, as in the apple data, and hence it is not known whether this phenomenon occurs in this tissue. With this possible exception, we conclude from these data and others, from both this laboratory and elsewhere (McArdle and Nehemias, 1956), that the preceding discussion of the relation between pectin content of irradiated and unirradiated apple tissue applies equally well to the results of our analysis of irradiated and unirradiated carrot tissues.

The data obtained from our analysis of beet tissue are presented in Table 3. Here the results are quite irregular, and not nearly as clear as those presented (in approximately the same detail) for apple tissue. Despite the high dose these tissues received, resulting in considerable softening, little or no evidence was obtained of consistent changes in the pectin constituents. The TD for softening of beets has been shown by Glegg *et al.* (1956) to be approximately 300 krad, a value considerably in excess of that found for apple tissue, and approximately double that of carrot tissue.

The changes in cellulose of irradiated McIntosh apples (Table 1) indicate progressive loss of viscosity with increased dose. The relatively large experimental variation in these cellulose determinations is a reflection of the rather complex procedure used for isolation of this component. Although both the intrinsic and specific viscosity values calculated for a given cellulose concentration show a decrease with increasing radiation, the decrease in the specific viscosity values calculated for a 0.15% cellulose solution appears to express this relationship best. The cellulose results presented for carrots (Table 2) indicate somewhat similar results, although, as with pectin, not to the marked extent in apple tissue. With beets, however, no consistent trend was obtained (Table 3).

Fig. 2 summarizes the over-all relationship between softening of irradiated apple cylinders and degradation of tissue pectins and cellulose. The TD for the degradation of pectins and cellulose is nearly identical

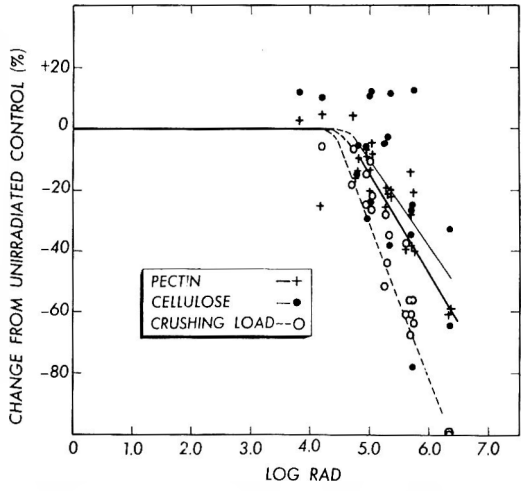


Fig. 2. Over-all relation between softening of irradiated apple tissues as measured by the crushing load and degradation of tissue pectins and cellulose as measured by changes in viscosity.

with the TD where the first measurable radiation-induced softening occurs. Pectin appears to be somewhat more susceptible to degradation by ionizing radiation than does cellulose. It will be noted that there is not a complete coincidence of the dose response of either pectin or cellulose degradation to that of tissue softening; however, it is possible that the additive effect of the two responses could account for the resultant softening.

This relationship is apparently not nearly as clear, however, with all plant tissues. Although measurable degradation of both pectins and cellulose of carrot tissues has been shown to occur in a manner similar to that of apple tissues, the softening of beets by irradiation does not seem to be as clearly accompanied by changes in these constituents. Whereas we have established that beets have a somewhat higher softening threshold value than that of the other two tissues examined, this is not sufficiently higher to explain the absence of the relationship. It is suspected that some additional factors, perhaps including changes in cell turgidity, are involved in the softening of plant tissue by gamma irradiation.

REFERENCES

Boyle, F. P., Z. I. Kertesz, R. E. Glegg, and M. A. Connor. 1957. Effects of ionizing radiations on plant tissues. II. Softening of

different varieties of apples and carrots. *Food Research* **5**, 372.

Brasch, A., and W. Huber. 1947. Ultra-short application time of penetrating electrons. A tool for sterilizing and preservation of food in the raw state. *Science* **105**, 112.

Glegg, R. E., and Z. I. Kertesz. 1957. Effect of gamma radiation on cellulose. *J. Polymer Sci.* **26**, 289.

Glegg, R. E., F. P. Boyle, L. W. Tuttle, D. E. Wilson, and Z. I. Kertesz. 1956. Effect of ionizing radiations on plant tissues. I. Quantitative measurements of the softening of apples, beets and carrots. *Radiation Research* **5**, 127.

Hannan, R. S. 1955. Scientific and technological problems involved in using ionizing radiations for the preservation of food. Food Investigation, Special Report No. 61, Her Majesty's Stationery Office, London. pp. 122–131.

Kertesz, Z. I. 1951. The pectic substances. Interscience Publishers, New York, N. Y. 628 pp.

Kertesz, Z. I., B. H. Morgan, L. W. Tuttle, and M. Lavin. 1956. Effect of ionizing radiations on pectin. *Radiation Research* **5**, 372.

Kertesz, Z. I., M. Eucare, and G. Fox. 1958. A study of apple cellulose. *Food Research* **24**, 14.

McArdle, F. J., and J. W. Nehemias. 1956. Effects of gamma radiation on the pectic constituents of fruits and vegetables. *Food Technol.*, **10**, 599.

McComb, E. A., and R. M. McCready. 1952. Colorimetric determination of pectic substances. *Anal. Chem.* **24**, 1630.

Morgan, B. H. 1955. Food radiation roundup: How different foods stand up. *Food Eng.* **27**, 44.

Moyer, J. C., D. E. Wilson, and D. B. Hand. (In preparation.) An instrument for evaluating the texture of fruits and vegetables.

Roberts, E. A., and B. E. Proctor. 1955. The comparative effect of ionizing radiations and heat upon the starch-containing cells of the potato tuber. *Food Research* **20**, 254.

Skinner, E. R., and Z. I. Kertesz. 1960. The effect of gamma radiation on the structure of pectin. An electrophoretic study. *J. Polymer Sci.* **47**, 99.

Wahba, I. J., D. F. Tallman, and L. M. Massey, Jr. 1963. Radiation-induced gelation of dilute aqueous pectin solutions. *Science* **139**, 1297.

Weihe, H. D., and M. Phillips. 1947. The quantitative estimation of hemicelluloses by direct isolation. *J. Agr. Research* **74**, 77.

Changes in Chicken Muscle Proteins During Aseptic Storage at Above-Freezing Temperatures[a]

A. W. KHAN AND L. VAN DEN BERG
National Research Council, Ottawa, Canada

(Manuscript received May 23, 1963)

SUMMARY

Analysis of chicken muscle proteins of 10-week-, 4-month-, and 8-month-old birds, stored under aseptic conditions at 0, 2, and 5°C, showed quantitative changes in the total extractable, myofibrillar, sarcoplasmic, and non-protein-nitrogen fractions during 7 weeks storage. Changes were small in the stroma-protein fraction, actomyosin fraction, and the myosin-adenosinetriphosphatase activity of the actomyosin fraction. The myosin fraction increased during storage except in breast muscle of 10-week-old birds. The sarcoplasmic-protein fraction decreased in the leg muscle of 10-week-old birds and the breast muscle of 4- and 8-month-old birds, but not in the breast muscle of 10-week-old birds. The non-protein-nitrogen fraction and the amount of protein-breakdown products increased in both breast and leg meat, irrespective of bird age. Proteolysis increased with storage time and temperature. The significance of proteolytic changes in quality deterioration is discussed, and the results are compared with those previously obtained for storage at below-freezing temperatures.

INTRODUCTION

Changes in chicken breast and leg muscle proteins during frozen storage were previously investigated (Khan *et al.*, 1963) as part of a wider study on the effects of freezing and frozen storage on poultry meat. The work has been extended to compare changes occurring during storage at above- and below-freezing temperatures. Microbial growth and oxidation, which in normal storage would not permit such a comparison, were minimized by storing the samples under aseptic conditions in nitrogen. This paper describes quantitative changes in myofibrillar, sarcoplasmic, stroma, and non-protein-nitrogen fractions of breast and leg muscle from chickens of different ages.

EXPERIMENTAL METHODS

Tests were made with meat from 10-week-, 4-month-, and 8-month-old chickens (male, Ottawa Meat Control Strain). Birds in each age group were obtained from a single flock, and 4–8 birds were used in each test. The birds were killed in the laboratory by cutting the jugular vein and carotid artery, bled for 2–3 min, scalded for 2 min at 53–54°C, plucked by hand, eviscerated, and aged for 24 hr in drained ice. Meat was deboned and dipped in chlortetracycline solution, 10 ppm, and sodium hypochlorite solution, 50 ppm. Each sample was placed in a sterilized Cry-O-Vac bag, and the bag was flushed with nitrogen. To obviate the effect of bird-to-bird variability, comparisons were made between two or four samples of breast and leg muscle of one bird stored at 0°C for different periods during maximum storage life of chicken meat (up to 7 weeks). To test the effect of storage temperature, some experiments were also made at 2°C and 5°C. Microbiological examination of samples at the time of analysis (van den Berg *et al.*, 1963a) showed no bacterial contamination in most cases. Results from experiments with samples found contaminated were discarded.

Preparation of samples, and extraction and fractionation techniques found most suitable for analysis of chicken muscle proteins (Khan, 1962), were used. In brief, extraction and fractionation of muscle proteins was carried out in KCl-borate buffer, pH 7.5 and ionic strength ($\Gamma/2$) 1.0. The non-dialyzable-nitrogen fraction soluble at $\Gamma/2 = 0.50$ and insoluble at $\Gamma/2 = 0.25$ is reported as actomyosin, that soluble at $\Gamma/2 = 0.25$ and insoluble at $\Gamma/2 = 0.08$ as myosin, and that soluble at $\Gamma/2 = 0.08$ as sarcoplasmic proteins. The non-protein-nitrogen fraction was obtained by precipitating proteins with trichloroacetic acid. Protein-breakdown products were estimated in the

[a] Paper presented at the Annual Meeting of the Institute of Food Technology, May 26–29, 1963, Detroit.

Contribution from the Division of Applied Biology, National Research Council, Ottawa 2, Canada. Issued as N.R.C. No. 7699.

non-protein-nitrogen fraction both by Folin-Ciocalteu reagent (Folin and Ciocalteu, 1927) and by ninhydrin reagent (Rosen, 1957) and were expressed in terms of tyrosine equivalent. Stroma-protein nitrogen (collagen and elastin) was determined in the unextractable-nitrogen fraction according to Lowry *et al.* (1941), and the myosin-adenosinetriphosphatase activity (ATPase activity) in the actomyosin fraction according to Khan *et al.* (1963). Losses of nitrogen in the liquid exuded from meat during storage were also determined. All analyses were made in duplicate.

RESULTS AND DISCUSSION

Changes in nitrogen extractability during storage at above-freezing temperatures depended on age of bird and kind of meat (breast or leg), but storage temperature (0, 2, and 5°C) had little effect on nitrogen extractability in breast muscle (Table 1). Nitrogen extractability did not change in breast and leg muscle of 10-week-old birds, increased slightly (up to 3% over fresh samples) in breast muscle of 4- and 8-month-old birds, and increased up to 14% in leg muscle of 8-month-old birds during storage. The increase in nitrogen extractability, particularly in leg muscle of 8-month-old birds, is not accounted for by the small increase in solubility of the stroma-protein fraction (Table 3).

Table 2 gives results of quantitative fractionation of extractable nitrogen into actomyosin, myosin, sarcoplasmic and non-protein-nitrogen fractions of breast and leg muscle of 10-week-old birds and breast muscle of 4- and 8-month-old birds stored at 0°C. The myosin fraction increased in all samples during storage except in breast muscle of 10-week-old birds, where it decreased slightly. In all tests, the quantitative changes in the actomyosin fraction were small. The sarcoplasmic-protein fraction decreased in leg muscle of 10-week-old birds and breast muscle of 4- and 8-month-old birds but not in breast muscle of 10-week-old birds. The non-protein-nitrogen fraction increased during storage in all these samples. This may indicate that sarcoplasmic proteins are broken down as a result of proteolysis. However, the decrease in sarcoplasmic nitrogen during storage (100–500 mg/100 g of muscle) could not be completely accounted for by loss of nitrogen in drip (30–90 mg N/100 g of muscle), nor by the increase of non-protein-nitrogen (30–170 mg N/100 g of muscle). Since an increase in the myosin fraction occurred without appreciable loss of the actomyosin fraction, an aggregation or an interaction between myosin and sarcoplasmic proteins is indicated. Bendall and Wismer-Pedersen (1962) have shown an interaction between actomyosin and denatured sarcoplasmic proteins with the loss of extractability at higher ionic strength in muscle from watery pork. In chicken muscle stored at 0°C, the gain of proteins in the myosin fraction occurred without the loss of extractability. The myosin-adenosinetriphosphatase activity of the actomyosin fraction did not change appreciably (Table 3), but this would not exclude the possibility of limited proteolysis in myofibrillar proteins (Gergely *et al.*, 1955; Mihalyi and Szent-Györgyi, 1953).

The amount of protein-breakdown products increased with storage time and temperature in both breast and leg meat (Table 4). The amount of Folin-Ciocalteu-

Table 1. Effect of storage time and temperature, kind of meat, and age of birds on extractability of muscle protein. (Values having the same superscript are for samples from the same bird).

Bird age	Kind of muscle	Storage temp. (°C)	Extractable nitrogen (% of total N)					
			Storage time (weeks)					
			0	1	2	3	4	5
10 weeks	breast	0	88.4[a]	88.3[a]	88.4[a]	.....	.....	88.3[a]
	leg	0	.....	78.3[b]	78.2[b]	.....	79.6[b]	78.8[b]
4 months	breast	0	88.1[c]	87.3[c]	.....	89.4[c]	90.3[c]	.....
		2	86.1[d]	87.2[e]	.....	86.0[d]	90.0[e]	.....
		5	85.3[f]	87.3[g]	.....	86.8[f]	90.9[g]	.....
8 months	breast	0	85.4[h]	85.7[h]	.....	88.7[h]	88.4[h]	.....
	leg	0	.....	61.3[j]	.....	60.8[k]	71.3[j]	75.1[k]

Table 2. Effect of storage time at 0°C and age of birds on muscle proteins (values having the same superscript are for samples from the same bird).

Protein fraction	Bird age	Kind of meat	Nitrogen (% of total extractable nitrogen) Storage time (weeks) 0	1	2	3	4	5	6	7
Sarcoplasmic	10 weeks	Breast	25.8[a]	26.4[b]	....	....	23.7[a]	....	....	25.0[b]
		Leg	....	29.6[e]	....	29.0[i]	....	23.0[i]	....	18.0[e]
	4 months	Breast	35.8[e]	36.5[f]	....	33.6[f]	....	25.3[e]	....	....
	8 months	Breast	39.0[g]	....	40.3[h]	....	....	26.0[g]	26.0[h]	....
Actomyosin	10 weeks	Breast	40.9[a]	40.4[b]	....	....	42.4[a]	....	....	41.0[b]
		Leg	....	43.8[e]	....	41.4[i]	....	43.0[d]	....	44.4[e]
	4 months	Breast	39.3[g]	40.5[f]	....	39.7[f]	....	41.0[e]	....	....
	8 months	Breast	36.6[g]	....	37.4[h]	....	....	36.2[g]	36.1[h]	....
Myosin	10 weeks	Breast	14.5[a]	15.0[b]	....	....	12.0[a]	....	....	12.0[b]
		Leg	....	10.5[e]	....	13.5[d]	....	15.0[d]	....	18.7[e]
	4 months	Breast	7.0[e]	6.0[f]	....	8.4[f]	....	12.1[e]	....	....
	8 months	Breast	2.7[g]	....	1.3[h]	....	....	11.7[g]	14.5[h]	....
Non-protein nitrogen	10 weeks	Breast	18.0[a]	17.3[b]	....	....	21.3[a]	....	....	21.8[b]
		Leg	....	16.0[e]	....	16.0[i]	....	17.9[d]	....	17.6[e]
	4 months	Breast	17.8[e]	16.5[f]	....	17.3[f]	....	18.8[e]	....	....
	8 months	Breast	19.3[g]	....	20.9[h]	....	....	24.3[g]	23.3[h]	....

reagent-positive materials (tyrosine, tryptophan, phenols, sulfhydryl compounds, and other reducing agents), increased by 100% in 4 weeks at 0°C and by the same amount in 3 weeks at 5°C, in both breast and leg muscle. The ninhydrin-positive materials increased by 30% at 0°C and by 60–70% at 5°C in 3 weeks of storage. A comparison of these results with those obtained by organoleptic tests on chicken meat stored under similar conditions (van den Berg *et al.*, 1963, 1964) shows that protein-breakdown products and odor scores increased concomitantly, and indicates that further investigation of the changes in the Folin-Ciocalteu-positive materials and in the ninhydrin-positive materials may suggest objective tests for quality changes in poultry.

A comparison of results in this paper with those obtained in a previous study on frozen chicken muscle (Khan *et al.*, 1963) shows a similarity in the accumulation of the protein-breakdown products only, but the rate at which the protein-breakdown products accumulated in meat stored at above-freezing temperatures was 20–30 times as fast as that in meat stored at below-freezing temperatures. The loss of protein solubility and denaturation of the actomyosin fraction with the loss of myosin-ATPase activity observed in meat stored at below-freezing temperatures and not in meat stored at above-freezing temperatures, appears to be a result of frozen storage.

ACKNOWLEDGMENT

The authors thank Mr. G. W. Daechsel and Mr. D. P. Driscoll for technical assistance.

REFERENCES

Bendall, J. R., and J. Wismer-Pedersen. 1962. Some properties of the fibrillar proteins of normal and watery pork muscle. *J. Food Sci.* **27**, 144.

Folin, O., and V. Ciocalteu. 1927. On tyrosine and tryptophane determination in proteins. *J. Biol. Chem.* **73**, 627.

Gergely, J., M. A. Gouvea, and D. Karibian. 1955. Fragmentation of myosin by chymotrypsin. *J. Biol. Chem.* **212**, 165.

Khan, A. W. 1962. Extraction and fractionation of proteins in fresh chicken muscle. *J. Food Sci.* **27**, 430.

Table 3. Change in stroma-protein fraction and myosin-adenosinetriphosphatase activity of actomyosin fraction after 4 to 5 weeks of storage at 0°C (values are averages of three samples).

Bird age	Kind of muscle	Storage time (weeks)	Stroma nitrogen (% of total N)		ATPase activity (μg of P released/mg N/min)	
			Fresh	Stored	Fresh	Stored
10 weeks	breast	5	10.0	9.8	240	230
10 weeks	leg	5	19.6	19.0	200	210
4 months	breast	4	10.6	9.3	190	180
8 months	breast	4	13.6	11.4	180	170
8 months	leg	5	25.7	23.4	.....	.....

Table 4. Effect of storage time and temperature, kind of muscle, and age of birds on accumulation of protein-breakdown products (values are averages of three samples).

Bird age	Kind of muscle	Storage temp. (°C)	Storage time (weeks)						
			0	1	2	3	4	5	6
			Folin-Ciocalteu-reagent-positive materials as tyrosine, mg/100 g of muscle						
10 weeks	breast	0	33.3	42.5	52.5	63.8	66.3	75.0	83.8
	leg	0	22.5	31.3	47.5	52.5	54.0	58.8	62.5
4 months	breast	0	29.4	31.3	40.7	.....	59.4	78.2	.....
	breast	2	29.4	31.3	42.0	.....	70.0	.....	.....
	breast	5	29.5	31.2	46.2	.....	71.9	.....	.....
8 months	breast	0	31.2	40.6	56.2	62.5	.....	65.0	67.5
	breast	5	34.2	51.2	61.8	67.3	.....	.....	.....
	leg	5	24.8	35.0	42.5	55.0	.....	.....	.....
			Ninhydrin-positive materials as tyrosine, g/100 g of muscle						
10 weeks	breast	0	1.21	.....	1.51	.....	1.67	.....	1.94
	leg	0	0.70	0.75	.....	0.97	.....	1.03	.....
4 months	breast	0	1.25	1.31	1.63	1.78	.....	2.34	.....
	breast	2	1.25	1.47	1.72	2.04	.....	.....	.....
	breast	5	1.25	1.56	1.94	2.10	.....	.....	.....
8 months	breast	0	1.15	1.25	1.78	2.15	.....	.....	.....
	leg	0	0.75	1.06	1.22	1.38	.....	.....	.....

Khan, A. W., L. van den Berg, and C. P. Lentz. 1963. Effect of frozen storage on chicken muscle proteins. *J. Food Sci.* **28**, 425.

Lowry, O. H., D. R. Gilligam, and E. M. Katevsky. 1941. The determination of collagen and elastin in tissues with results obtained in various normal tissues from different species. *J. Biol. Chem.* **139**, 795.

Mihalyi, E., and A. G. Szent-Györgyi. 1953. Trypsin digestion of muscle proteins. I. Ultracentrifuge analysis of the process. *J. Biol. Chem.* **201**, 189.

Rosen, H. 1957. A modified ninhydrin colorimetric analysis for amino acids. *Arch. Biochem. Biophys.* **67**, 10.

van den Berg, L., A. W. Khan, and C. P. Lentz. 1963. Biochemical and quality changes in chicken meat during storage at above-freezing temperatures. *Food Technol.* **17**, 91.

van den Berg, L., C. P. Lentz, and A. W. Khan. 1964. Changes in quality, water-holding and ion-binding properties of chicken meat during above-freezing storage under aseptic conditions. *Food Technol.* In press.

The Effect of Peroxidase on Anthocyanin Pigments[a]

RUTH GROMMECK AND P. MARKAKIS
Department of Food Science, Michigan State University
East Lansing, Michigan

(Manuscript received June 24, 1963)

SUMMARY

Purified cyanidin 3-gentiobioside, cyanidin 3-rhamnoglucoside, and pelargonidin 3-glucoside are decolorized by horseradish peroxidase in acetate buffer. Among the optimum conditions for the reaction are pH 4.5–5.5, a hydrogen peroxide concentration of 10^{-4} to $10^{-3}M$, and a temperature of 60–70°C. A k_1 value equal to $110 \times 10^{1} M^{-1} \times sec^{-1}$ was estimated for the reaction horseradish peroxidase-hydrogen peroxide-pelargonidin 3-monoglucoside at pH 4.7.

INTRODUCTION

Anthocyanins are generally unstable pigments. Several physical and chemical factors have been found to influence the rate of degradation of these pigments in fruit products and model systems (Nebesky *et al.*, 1949; Sondheimer and Kertesz, 1952; Markakis *et al.*, 1957).

Studies on the enzymatic degradation of anthocyanins have been rather limited. In 1921 Nagai observed that, in the presence of H_2O_2, aqueous extracts of scarlet poppies were decolorized by a crude enzyme preparation obtained from the hypocotyls of soybean seedlings. Huang (1955) showed that enzyme extracts of *Aspergillus* hydrolyzed the anthocyanins of blackberries to anthocyanidin and sugar, with subsequent spontaneous decolorization of the aglycone. Bayer and Wegmann (1957) obtained from the leaves of *Coleus hybridus* a "cyaninoxidase" that rapidly decolorized the anthocyanin of red roses in the presence of catechol. More recently van Buren *et al.* (1959) and Scheiner (1960) tried to isolate an enzyme that specifically decolorizes anthocyanins, and Peng and Markakis (1963) reported on the effect of phenolase on anthocyanins.

This investigation was made to study the effect of a typical peroxidase, the horseradish peroxidase, on purified anthocyanin pigments under controlled conditions. Peroxidase activity has been found in many plant tissues, and the specificity of the peroxidase-hydrogen peroxide complex toward hydrogen donors is known to be quite low (Joslyn, 1949). Peroxidase activity has also been detected in red tart cherries (Bedford, 1962), one of the two kinds of fruits used as sources of purified anthocyanins in this study.

MATERIALS AND METHODS

Preparation of anthocyanins. Two cyanidin and one pelargonidin glycosides were prepared from red tart cherries and strawberries, respectively.

Fresh or frozen pitted Montmorency cherries were placed in boiling 95% (v/v) ethanol in such a proportion as to achieve a 70–75% (v/v) final ethanol concentration. The mixture was boiled for 4–5 min and allowed to cool. This treatment extracted the anthocyanins, precipitated the pectins, and inactivated the enzymes. The extract was passed through a milk filter disc, and the filtrate was concentrated under 2–3 cm Hg of pressure in a rotary film evaporator thermostatically controlled at 37°C. The concentrate was applied to a column of Dowex 50 W-X8 resin (100–200-mesh, H+ form), large enough to retain almost all of the red pigment. The column was then washed with water, and the red pigment was eluted with a mixture of methanol (98 vol) and conc. HCl (2 vol). The eluate, after concentration in the vacuum evaporator, was applied as a narrow band onto Whatman 3 MM paper. The paper was irrigated ascendingly with 1*N* acetic acid in a chromatography cabinet for 15–20 min. Then the two cherry anthocyanins appeared as well-separated zones, which were cut off and eluted with methanol containing a trace of conc. HCl. The eluates of the separated pigments were again concentrated *in vacuo*, and they were further purified by zone electrophoresis on cellulose powder

[a] This research was supported in part by the U. S. Public Health Service (Grant RG 9025). This paper has been assigned Journal Article No. 3182 by the Michigan Agricultural Experiment Station.

with 1*N* acetic acid as the electrolyte (Markakis, 1960). The absorption spectra in the ultraviolet and visible regions, the paper chromatographic behavior, and the sodium carbonate test of the purified pigments agreed with those observed by Li and Wagenknecht (1956) for cyanidin-3-gentiobioside (C-3-Gb) and cyanidin-3-rhamnoglucoside (C-3-Rg).

The major strawberry anthocyanin, pelargonidin-3-monoglucoside (P-3-G), was purified by the same general method used for the cherry pigments, except that *n*-butanol–acetic acid–water (40:10:12.5, v/v) was employed as solvent in the paper chromatographic step, and the irrigation lasted 16 hr.

The enzyme. A lyophilized soluble preparation of horseradish peroxidase (HRP), supplied by General Biochemicals, Inc., was used. We found the degree of purity (A_{403}/A_{275}) of the preparation to be 1.00.

The reaction. Four factors likely to affect the reaction between HRP, H_2O_2, and anthocyanin were studied: pH, H_2O_2 concentration, enzyme concentration, and temperature of incubation. For study of the pH effect, 3.0 ml of a $10^{-1}M$ solution in acetic acid and $10^{-3}M$ in H_2O_2 were mixed with 0.5 ml anthocyanin solution, and the pH of the mixture was adjusted to the desired level by adding 1*N* NaOH from a 1-ml graduated pipette and using a combination glass-silver-silver chloride electrode. Sufficient water was added to make the total volume 4.5 ml. One ml of this mixture was transferred to 3.0 ml of 1*M* citric acid solution to which 0.1 ml of a $10^{-3}M$ KCN solution had just been added. The absorbancy of the transferred portion, obtained 15 min after mixing it with the citric-cyanide solution, was used as a measure of the zero-time concentration of anthocyanin. Immediately after the first transfer, 0.1 ml of enzyme solution was added to the magnetically stirred reaction mixture, a timer was started, and at 30-sec intervals 1-ml portions were transferred to a citric-cyanide solution identical with the one used for zero time. Fifteen minutes after each transfer the absorbancy was measured with a Beckman DU spectrophotometer set at the following wavelengths for the different pigments: 520 mμ for C-3-Gb, 515 mμ for C-3-Rg, and 500 mμ for P-3-G. In the study of the other factors, 0.1*M* acetate buffer of pH 5.0 was used. Since H_2O_2 alone can oxidize anthocyanins, reaction mixtures without peroxidase were prepared and the destruction of pigment due to the single effect of H_2O_2 was subtracted from that of the combined peroxidase-peroxide decolorization. The differences were plotted against time, and the initial rate of the reaction was estimated by sight-fitting of the tangent at the origin.

RESULTS AND DISCUSSION

The data on the effect of peroxidase on cyanidin 3-gentiobioside are graphically presented in Figs. 1, 2, 3, and 4; those on cyanidin 3-rhamnoglucoside and pelargonidin 3-glucoside are given in Tables 1 and 2, respectively. These data are the average of two initial reaction rate values not differing from each other by more than ±5%.

These results indicate that the anthocya-

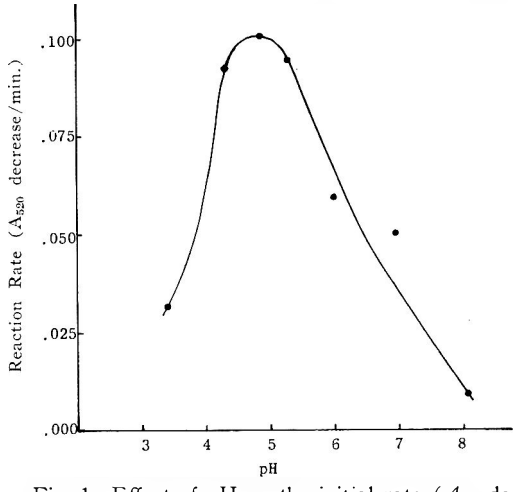


Fig. 1. Effect of pH on the initial rate (A_{520} decrease per min) of the horseradish peroxidase, H_2O_2, cyanidin 3-gentiobioside reaction (2.8×10^{-4} mg enzyme per ml of reaction mixture; $6.5\times10^{-4}M$ H_2O_2; 26°C).

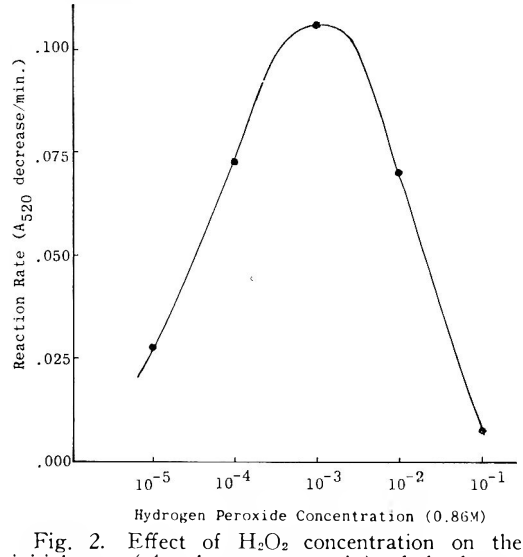


Fig. 2. Effect of H_2O_2 concentration on the initial rate (A_{520} decrease per min) of the horseradish peroxidase, H_2O_2, cyanidin 3-gentiobioside reaction (2.8×10^{-4} mg enzyme per ml of reaction mixture; pH 5.0; 26°C).

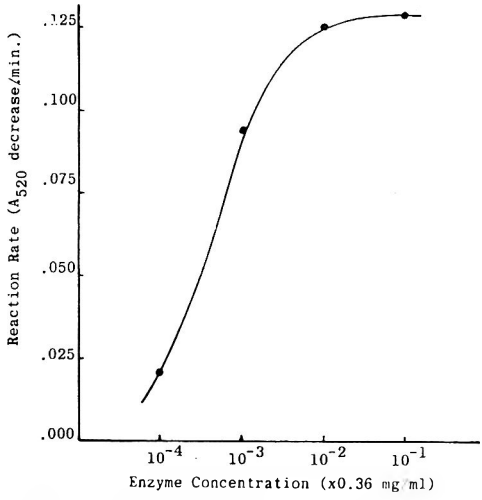


Fig. 3. Effect of enzyme concentration on the initial rate (A_{520} decrease per min) of the horseradish peroxidase, H_2O_2, cyanidin 3-gentibioside reaction ($8.6\times10^{-4}M$ H_2O_2; pH 5.0; 26°C).

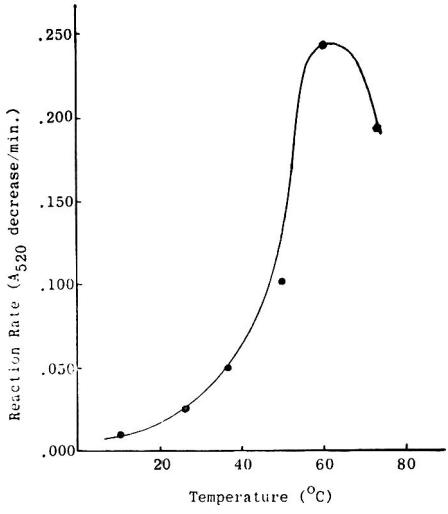


Fig. 4. Effect of temperature on the initial rate (A_{500} decrease per min) of the horseradish peroxidase, H_2O_2, cyanidin 3-gentiobioside reaction (2.8×10^{-5} mg enzyme per ml of reaction mixture; $8.6\times10^{-4}M$ H_2O_2; pH 5.0).

nins tested are degraded faster at pH 4.5–5.5 than at other pH levels under the specified conditions. Chance (1949b) found that the velocity of the reaction of the peroxide-peroxidase Complex II with some hydrogen donors is affected only slightly by pH in the region 3.5–5.3, and in cases in which the rate is definitely changed with pH, either the donor molecule or the oxidation products change with pH. The structure of anthocyanins is known to change with pH, and this perhaps explains the effect of pH on their oxidation rate by peroxidase.

The concentration on H_2O_2 also appears to affect the initial rate of anthocyanin destruction by peroxidase. The rate increases with H_2O_2 concentration up to a certain level (10^{-4} to $10^{-3}M$), above which the H_2O_2 probably becomes deleterious to the enzyme.

Table 1. Effect of various factors on the initial rate (A_{615} decrease per min) of the horseradish peroxidase, H_2O_2, cyanidin-3-rhamnoglucoside reaction in acetate buffer.

pH	2.3	4.1	4.8	5.1	6.8	enzyme 2.8×10^{-4} mg/ml;
Reaction rate	.056	.134	.152	.096	.050	H_2O_2 $6.5\times10^{-4}M$; 26°C
H_2O_2 concn. (M)		8.6×10^{-2}	8.6×10^{-3}	8.6×10^{-4}	8.6×10^{-5}	enzyme 2.8×10^{-4} mg/ml;
Reaction rate		.022	....	.150	.090	pH 5.0; 26°C
Enzyme concn. (mg/ml)		2.8×10^{-3}	2.8×10^{-4}	2.8×10^{-5}	2.8×10^{-6}	H_2O_2 $8.6\times10^{-4}M$; pH 5.0; 26°C
Reaction rate		.152	.148	.134	.206	

Table 2. Effect of various factors on the initial rate (A_{500} decrease per min) of the horseradish peroxidase, H_2O_2, pelargonidin-3-glucoside reaction in acetate buffer.

pH	4.2	4.8	5.1	5.5	6.7		enzyme 2.8×10^{-5} mg/ml;
Reaction rate	.008	.016	.027	.024	.012		H_2O_2 $6.5\times10^{-4}M$; 26°C
H_2O_2 concn. (M)	8.6×10^{-2}	8.6×10^{-3}	8.6×10^{-4}	8.6×10^{-5}			enzyme 2.8×10^{-5} mg/ml;
Reaction rate	.000	.021	.030	.022			pH 5.0; 26°C
Temp. (°C)	26	42	50	60	70	74	enzyme 2.8×10^{-5} mg/ml;
Reaction rate	.030	.060	.084	.220	.280	230	H_2O_2 $8.6\times10^{-4}M$; pH 5.0

The effect of the enzyme concentration is rather typical inasmuch as the reaction rate increases with concentration and then levels off.

Temperature definitely affects the reaction rate, which increases rapidly as the temperature is raised, reaches a maximum in the region between 60 and 70°, and then falls. This high-temperature optimum is compatible with the well known thermostability of peroxidase.

On the basis of this work, anthocyanins should be added to the already long list of substances oxidized by the peroxidase-peroxide complexes (Joslyn, 1949). Since Chance (1949b) has measured the peroxidase oxidizability of various hydrogen donors in terms of the kinetic constant k_4, it was deemed desirable to estimate this constant for at least one of the available anthocyanins.

Calculation of k_4 for pelargonidin 3-glucoside. The rate-determining reactions in a system containing peroxidase (E), hydrogen peroxide (S), and a hydrogen donor (AH) are:

$$E + S \xrightarrow{k_1} ES \qquad [1]$$

$$ES + AH \xrightarrow{k_4} E+A+SII \qquad [2]$$

k_1 measures the velocity at which complex ESI is formed; in the presence of a hydrogen donor this complex is transformed to $ESII$ as rapidly as it is produced and $ESII$ oxidizes the donor. k_4 is taken as a measure of peroxidase activity toward a particular donor. Chance (1949a,b) was able to calculate k_4 from the kinetics of the disappearance of substrate, and also from the rate of oxidation of some donors on the basis of the equation:

$$k_4 = \frac{dx}{dt} \frac{1}{p_{\max}\,[AH]} \qquad [3]$$

in which $\frac{dx}{dt}$ is the rate of disappearance of H_2O_2 or the rate of oxidation of AH, $p_{\max}$ is the maximum concentration of enzyme-substrate complex during the cycle and $[AH]$ is the initial concentration of donor.

Since the molecular extinction coefficient of P-3-G is known (Markakis *et al.*, 1957), this pigment was chosen for the calculation of k_4. The horseradish peroxidase concentration was determined by the method of Maehly and Chance (1954) on a shipment of commercial enzyme received after most of this research had been done. A reaction system was set up: $2.2\times10^{-8}M$ in HRP, $5.3\times10^{-5}M$ in anthocyanin, $6.5\times10^{-4}M$ in H_2O_2, and $6.5\times10^{-2}M$ in acetate buffer, pH 4.7, the pH at which Chance (1949b) had obtained k_4 values for other donors. The initial rate of the A_{500} decrease, corrected for the single effect of H_2O_2, was used to calculate dx/dt, and the following value was obtained:

$$k_4 = 1.3\times10^{-6}.\ \frac{1}{2.2\times10^{-8}\times5.3\times10^{-5}} = 110\times10^4$$

This value is between the k_4 ($M^{-1}\times\sec^{-1}\times10^{-4}$) for hydroquinone (250) and catechol (230) on one hand, and those for pyrogallol (21) and ascorbic acid (1.2) on the other. However, in a mixture of P-3-G and ascorbic acid, the anthocyanin is not oxidized first. On the contrary, ascorbic acid seems to protect the pigment. In a test in which ascorbic acid was added to the enzymatic system at the concentration of $10^{-4}M$, the initial rate of anthocyanin oxidation was 10 times as small. This can probably be explained by the "cyclic" process that Chance proposed for the retardation of guaiacol oxidation by peroxidase in the presence of ascorbic acid. That is, the ascorbic acid reduces the primary oxidation product of the anthocyanin, which is again oxidized enzymatically, and so on.

REFERENCES

Bayer, E., and K. Wegmann. 1957. Enzymatischer Abbau von Anthocyanin. *Z. Naturforsch.* **12b**, 37.

Bedford, C. L. 1962. Private communication.

Chance, B. 1949a. The properties of enzyme-substrate compounds of horseradish peroxidase and peroxides. III. The reaction of complex II with ascorbic acid. *Arch. Biochem.* **24**, 389.

Chance, B. 1949b. The properties of enzyme-substrate compounds of horseradish peroxidase and peroxides. IV. Effect of pH upon the rate of complex II with several acceptors and its relation to their oxidation-reduction potential. *Arch. Biochem.* **24**, 410.

Huang, H. T. 1955. Decolorization of anthocyanins by fungal enzymes. *J. Agr. Food Chem.* **3**, 141.

Joslyn, M. A. 1949. Enzyme activity in frozen vegetable tissue. *Advances in Enzymol.* (F. F. Nord, ed.) **9**, 613.

Li, K. C., and A. C. Wagenknecht. 1956. The anthocyanin pigments of sour cherries. *J. Am. Chem. Soc.* **78**, 979.

Maehly, A. C., and B. Chance. 1954. Assay of catalases and peroxidases. *In* "Methods of Biochem. Analysis." (D. Glick, ed.) **1**, 357.

Markakis, P. 1960. Zone electrophoresis of anthrocyanins. *Nature* **187**, 1092.

Markakis, P., G. E. Livingston, and C. R. Fellers. 1957. Quantitative aspects of the strawberry pigment degradation. *Food Research* **22**, 117.

Nagai, I. 1921. A genetico-physiological study on the formation of anthocyanins and brown pigments in plants. *J. Coll. Agr. Tokyo Imp. Univ.* **8**, 2.

Nebesky, E. A., W. B. Esselen, and J. I. McConnel. 1949. Stability of color in fruit juices. *Food Research* **14**, 261.

Peng, C. Y., and P. Markakis. 1963. Effect of phenolase on anthocyanins. *Nature* **199**, 597.

Scheiner, D. M. 1960. The enzymatic decolorization of anthocyanin pigments. Thesis. Cornell Univ. Dissertation Absts. 1961. **21**, 2882.

Sondheimer, E., and Z. I. Kertesz. 1952. The kinetics of the oxidation of strawberry anthocyanin by H_2O_2. *Food Research* **17**, 288.

Van Buren, J. P., D. M. Scheiner, and A. C. Wagenknecht. 1959. Newly discovered enzyme may cause color loss in small fruits. *N. Y. Agr. Expt. Sta. Farm Research Bull.* **25**, 15.

Postharvest Metabolism of Green Peas (*Pisum sativum*) with Special Reference to Glutamic Acid and Related Compounds

C. ERIKSSON AND E. VON SYDOW
Swedish Institute for Food Preservation Research (SIK), Göteborg 16, Sweden

(Manuscript received May 3, 1963)

SUMMARY

An investigation was made of postharvest variations in the concentrations of glutamic acid, γ-aminohutyric acid, and aspartic acid in green peas that were stored in pods, vined but not damaged, and vined and damaged by mechanical pressure. Oxygen consumption and carbon dioxide production by damaged peas were determined, and the correlation of these values with γ-aminobutyric acid production is discussed. C^{14}-labeled glutamic acid, glutamine, α-ketoglutaric acid, and aspartic acid were added to the peas, and the distribution of radioactivity after a short time was analyzed. The approximate distribution of proteolytic activity, glutamic acid decarboxylase, and glutamic acid–oxalacetic acid transaminase was determined, and was correlated with the biochemical effect of damage.

INTRODUCTION

Glutamic acid is said to improve the natural flavor of certain foods. The effect of added glutamate to various foods has been described (Sjöström and Crocker, 1948; Norton *et al.*, 1952; Hanson *et al.*, 1960). Glutamate added to fresh vegetables harvested under conditions optimum from the flavor point of view has no effect (van Duyne *et al.*, 1957). The concentration of free L-glutamic acid in unblanched green peas decreases rapidly during one day's storage, whereas the concentration remains unchanged in blanched peas stored under the same conditions. This indicates the removal of L-glutamic acid by enzyme activities (Hac *et al.*, 1949).

On the plant, the concentration of free L-glutamic acid in green peas also decreases during ripening, when protein is being synthesized (Danielsson, 1952). The proteins of ripe peas, especially the globulins, are very rich in L-glutamic acid, vicilin containing 22.7% and legumin 30.1% (Danielsson and Lis, 1952). However, the amino acid composition of protein in ripe peas differs from that in unripe peas, the latter being richer in arginine but poorer in essential amino acids. Also, different-sized unripe peas differ in amino acid composition, the smaller ones being even poorer in essential amino acids (Schuphan and Postel, 1960).

Since L-glutamic acid occupies a key position in the metabolism of the living cell, its concentration can change in several ways. Factors such as temperature, gas phase, aerobic and anaerobic conditions, and degree of maturity are important for the balance of the L-glutamic acid metabolism. Green peas harvested for canning or deep-freezing are not ripe from a physiological point of view. The harvest means a drastic and sudden interruption of the ripening process, in which the pea, the pod, and the rest of the plant take part. This means that the time and treatment of the peas between harvest and stabilization are critical.

Proteolytic enzymes in peas have been investigated (Mergentime and Wiegand, 1946; Soedigdo and Gruber, 1960). The activity has been shown to be higher in unripe peas than in ripe peas (Danielsson, 1951).

For comparison, it might be mentioned that the proteolytic activity is higher in damaged wheat than in undamaged, whereby the concentrations of threonine, alanine, and γ-aminobutyric acid are increased, whereas that of L-glutamic acid is not. Deterioration of wheat is correlated with this increase of γ-aminobutyric acid at the expense of L-glutamic acid (Linko, 1960).

γ-Aminobutyric acid has also been identified in peas (Auclair and Maltais, 1952),

and it can be produced by enzymatic decarboxylation of L-glutamic acid. This enzyme occurs in higher plants, including peas (Schales *et al.*, 1946). Other L-glutamic acid enzymes are L-glutaminase and L-glutamic-oxalacetic acid transaminase (Stumpf, 1951), L-glutamic acid dehydrogenase.

In this work the postharvest changes of the concentration of L-glutamic acid, γ-aminobutyric acid, and L-aspartic acid in green peas stored in the pod, vined both damaged and undamaged, were studied. The oxygen consumption and carbon dioxide production in damaged and undamaged peas were measured and correlated with the γ-aminobutyric acid production. To trace the main steps for the production and removal of L-glutamic and L-aspartic acid, labeled L-glutamic acid, L-glutamine, L-aspartic acid, and α-ketoglutaric acid were added to damaged and undamaged peas. The distribution of radioactivity after a short time was analyzed. The distribution of certain enzymes among different parts of the pea fruit was estimated to assess the effect of damage on the enzyme substrate contact.

MATERIALS AND METHODS

Peas. Green peas (*Pisum sativum* sensu amp. (L) Govorov var. *pachylobum*, the wrinkled type), medium size, were collected from plants in southern Sweden at a tenderometer value of 104.

Chemicals. All amino acids and other acids were of pro analysi quality or chromatographically pure. L-Glutamic acid C^{14} specific activity 23.3 μC/mg, L-aspartic acid C^{14} specific activity 33.3 μC/mg, and α-ketoglutaric acid C^{14} specific activity 16.1 μC/mg were purchased from Radiochemical Centre, Amersham Bucks., England, and L-glutamine C^{14} specific activity 15.8 μC/mg from California Corporation of Biochemical Research, Los Angeles, Calif., USA. All compounds — uniformly labeled—were dissolved in sterile water before use. L-Glutamic acid decarboxylase, *Escherichia coli* ATCC 11246 specific activity 300 μC/mg for manometric determination of L-glutamic acid (Gale, 1957) was purchased from Worthington Biochemical Company, Freehold, N. J., USA.

L-Aspartic acid and γ-aminobutyric acid were determined after one hour's paper electrophoretic separation of 10-μl samples in 0.05*M* pyridinum acetate buffer at pH 4.2. The dry papers were dipped in ninhydrin solution (1 g ninhydrin, recrystallized, in acetone, acetic acid, and water 180 : 10 : 10) and heated for 30 min at 80°C, after which they were left for 15 min at room temperature. The separation curve was drawn by a scanning photometer (Analytrol, Beckman-Spinco Company) with 600-mμ interference filter. The area under the curves corresponding to L-aspartic acid and γ-aminobutyric acid was taken as a measure of the amount of the acids.

EXPERIMENTAL

Variation in L-glutamic acid, γ-aminobutyric acid and L-aspartic acid. Freshly harvested peas were: a) stored in the pods. After 0, ½, 1, 2, and 8 hours, samples were taken out to proceed according to b) or c); b) aseptically vined by hand and stored in a glass jar with a loose lid; c) aseptically vined by hand and rolled between glass plates under moderate pressure to cause slight damage (the skin was not ruptured) and stored with shaking at a low rate.

Samples of 15 g were withdrawn at 0, ½, 1, 2, 4, and 8 hr. The samples were immediately frozen and stored in liquid nitrogen until analysis. The peas were homogenized together with solid carbon dioxide and extracted with 80% alcohol. Alcohol was then removed by extraction with chloroform. The water phase containing the amino acids was freeze-dried and dissolved in 10 ml of pyridinium acetate buffer, pH 4.2, just before analysis. Samples were also taken for determination of nondialyzable bound and total free amino acids.

Respiration experiment. To measure respiration, peas were taken directly from the plant and aseptically damaged as before and transferred to Warburg flasks (total volume of 110–115 ml each).

Tracer experiment. Four μl of radioactive solution corresponding to 40 mμC and not more than 3 μg substance were injected in peas still attached to the pod. After injection, some peas were left in the pod whereas others were removed, damaged, and stored in a glass beaker. Thirty minutes after injection, all the labeled peas were frozen in liquid nitrogen and extracted as described.

After electrophoretic separation of 10 μl extract, the papers were cut and the radioactivity measured on dried water extracts of each piece. The extract from the L-aspartic C^{14} experiment was, in addition, tested for radioactive oxalacetic acid by treating the water extracts from the proper parts of a few papers after electrophoresis with dinitrophenyl hydrazine. The dinitrophenyl hydrazones obtained were separated chromatographically on Whatman 4 in *n*-butanol–glacial acetic acid–water (4 : 1 : 5). The paper was cut, and each piece was extracted with chloroform. Each extract was evaporated on planchets, and the radioactivity was measured. The time to obtain 10.000 counts was in all experiments registrated on a paper strip in an automatic β-ray counting system

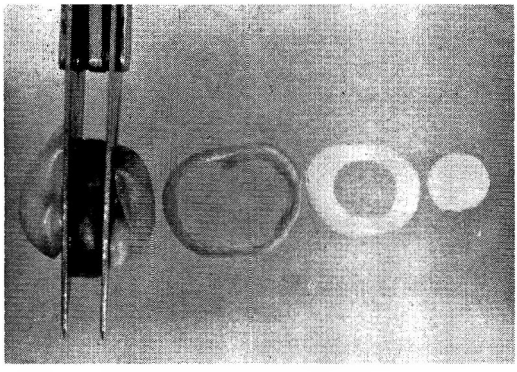


Fig. 1. From the left: slicing of the pea, skin, outer and inner part of cotyledon.

(Frieseke-Hoephner, Germany). The experiments were run in duplicate.

Enzyme localization. Frozen peas were cut with a double knife (Fig. 1) through the center parallel to the plane of division of the cotyledon, and the latter was subdivided. The slices, thickness 2.5 mm, were separated and thoroughly washed in distilled water and weighed.

Proteolytic activity was measured in 0.1*M* phosphate buffer, pH 6.8, as the increase in absorption at 570 mμ of ninhydrin-positive compounds soluble in 5% trichloro-acetic acid after ½, 1, and 2 hr.

L-Glutamic acid decarboxylase was determined as the increase in γ-aminobutyric acid concentration at 37°C in 0.1*M* L-glutamic acid in 10 ml 0.1*M* phosphate buffer containing 10 μg pyridoxalphosphate per ml. A parallel control run contained only slices and buffer. L-glutamic acid-oxalacetic acid transaminase was determined as the increase in L-glutamic acid concentration when slices were incubated at 37°C in 0.1*M* L-aspartic acid and 0.1*M* α-ketoglutaric acid in 10 ml 0.1*M* phosphate buffer containing 10 μg pyridoxalphosphate per ml at 37°C. A parallel control run contained only slices and buffer. Samples were withdrawn for analysis at ½, 1, and 2 hr.

RESULTS

In peas stored in their pods, the concentration of L-glutamic acid increases during the first 2 hr. A very slight increase in γ-aminobutyric acid concentration also occurs. The L-aspartic acid concentration shows a small but clear decrease (Table 1). One can see that in peas taken out of their pods the L-glutamic acid concentration increases once again during the first 2 hr after vining (Table 2) and then decreases again when the peas have been stored for a short time in pods before vining, but remains constant when prestored in pods for 2–8 hr. L-Aspartic acid shows the opposite picture. Its concentration decreases and passes a minimum value when prestorage time in pods is short, and decreases continuously when this time is long.

Table 1. Variations in the concentrations of L-glutamic acid, L-aspartic acid, and γ-aminobutyric acid in peas stored in pods.

Hr in pod	μ mole per g fresh weight		
	Glutamic acid	Aspartic acid	γ-Aminobutyric acid
0	7.8	3.5	0.9
0.5	8.7	2.7	1.3
1	9.4	3.1	1.0
2	10.1	2.8	1.0
8	10.6	1.9	1.3

Damaged peas behave like undamaged ones regarding L-glutamic acid, whereas the concentration of L-aspartic acid falls to zero at higher rates when prestored for a long time in pods (Table 2). Most striking is the behavior of γ-aminobutyric acid (Table 2). The concentration of this acid is low in fresh peas, but increases when vined peas are stored. If the peas are damaged, the rate of production is very much increased, and its concentration then equals that of L-glutamic acid in a few hours, irrespective of the time the peas have been stored in their pods before vining.

Another well known fact is that oxygen consumption is lowered and carbon dioxide production is increased in damaged peas, but the increase in carbon dioxide production is much greater than that corresponding to decarboxylation of L-glutamic acid (Table 3).

The main pathways for production and removal of, especially, L-glutamic acid can be seen from the tracer experiments. Radioactivity from labeled L-glutamic acid is transferred mainly to γ-aminobutyric acid among the substances soluble in 80% alcohol (Fig. 2). There was also much radioactivity transferred to a nondialyzable fraction, which after hydrolysis was found to contain active L-glutamic acid. The transfer of radioactive L-glutamic acid to γ-aminobutyric acid was more abundant in the damaged peas.

The radioactivity from L-glutamine is very rapidly transferred to active L-glutamic acid (in damaged peas this effect is even greater), and activity also occurs in γ-aminobutyric acid (Fig. 3-a,b). The same picture is obtained when α-ketoglutaric acid C^{14} is added to the peas. Radioactivity is transferred to both L-glutamic acid and γ-aminobutyric acid (Fig. 3-c).

Radioactivity from L-aspartic acid C^{14} is transferred to stronger acid compounds (Fig. 3-d), and, as expected, radioactivity is found in oxalacetic acid chromatographed as dinitrophenylhydrazone.

Table 2. Variation of the concentrations of L-glutamic acid, L-aspartic acid, and γ-aminobutyric acid in peas after prestorage in pods and vining. UD = undamaged, D = damaged peas.

Hr in pod	Hr after vining	μ mole per g fresh weight					
		Glutamic acid		Aspartic acid		γ-Aminobutyric acid	
		UD	D	UD	D	UD	D
0	0	7.8	....	3.5	....	0.9	....
	0.5	9.3	9.2	3.2	2.0	1.0	1.5
	1	9.6	9.6	2.5	1.5	1.2	1.8
	2	11.0	10.7	1.2	0.0	1.0	3.1
	4	10.1	10.5	2.1	0.0	1.2	5.1
	8	9.8	10.2	2.3	0.0	3.2	10.1
0.5	0	8.7	....	2.7	....	1.3	....
	0.5	9.2	9.1	2.7	1.9	1.3	1.6
	1	11.1	10.1	1.6	1.0	1.3	1.6
	2	10.4	11.7	2.0	0.0	1.3	3.5
	4	10.6	12.7	2.4	0.0	1.2	6.2
	8	9.4	12.1	2.1	0.0	3.9	12.6
2	0	10.1	....	2.8	....	1.0	....
	0.5	11.1	11.4	2.7	0.0	1.3	1.8
	1	11.0	12.5	2.5	0.0	1.2	3.2
	2	12.3	13.1	2.1	0.0	1.8	4.6
	4	12.7	13.3	....	0.0	2.3	7.2
	8	12.7	14.6	0.0	0.0	3.7	11.5
8	0	10.6	....	1.9	....	1.3	....
	0.5	11.0	8.2	....	....	....	....
	1	11.3	10.6	1.6	0.0	1.4	2.3
	2	11.4	11.9	1.1	0.0	1.0	3.5
	4	11.5	10.7	1.6	0.0	1.2	4.9
	8	12.1	11.4	1.5	0.0	1.9	10.1

Table 3. Oxygen consumption and carbon dioxide production in undamaged and damaged peas.

Min	Undamaged			Damaged		
	μ mole/g fresh weight			μ mole/g fresh weight		
	O_2	CO_2	γ-Aminobutyric acid	O_2	CO_2	γ-Aminobutyric acid
0	0	0	0	0	0	0
10	2.1	2.1	....	1.8	2.9	....
20	3.8	3.6	....	3.2	6.1	....
40	7.1	6.7	....	5.6	12.3	....
60	10.0	9.3	<0.1	8.2	20.5	1.2

In general, the actual enzyme activities are located in the skin and outer parts of the cotyledon (Fig. 4, 5, 6). Most striking in this respect is L-glutamic acid decarboxylase. The water used to wash the pea slices contained no such enzyme activities.

DISCUSSION

Material, mainly sucrose, is transported to peas in pods on the plant. During ripening the concentrations of free amino acids decrease. L-Glutamic acid and L-aspartic acid tend to decrease when vining follows soon after harvesting; L-glutamic acid, after passage of a maximum value. It has been proposed that a hormone controls the ripening process as long as the pea is attached to the plant (Wager, 1962). Most interesting, however, is the effect of even slight damage to the pea. Oxygen consumption decreases and carbon dioxide production

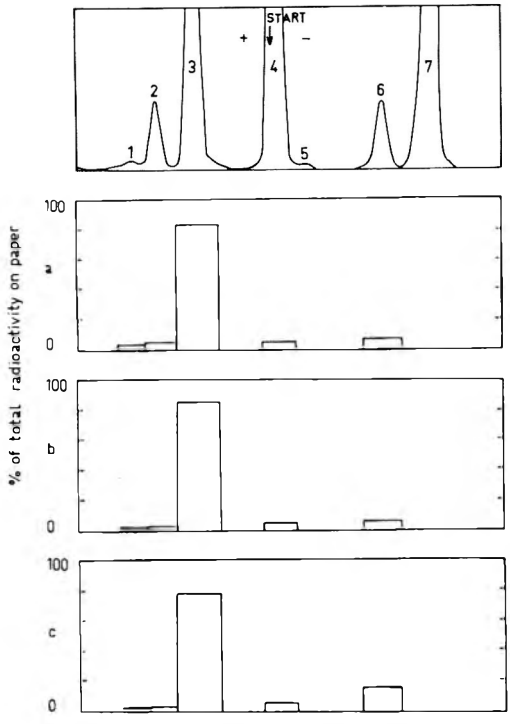


Fig. 2. Distribution of radioactivity 30 min after injection of L-glutamic acid C^{14} in peas
a) stored in pod
b) vined undamaged
c) vined damaged.
Peaks. 1. Aspartic acid
2. Unknown
3. Glutamic acid
4. Neutral amino acids
5. Unknown
6. γ-Aminobutyric acid
7. Basic amino acids.

increases markedly. This indicates that the metabolism in the peas becomes anaerobic. Undamaged peas have skins that are dry and waxy, but peas damaged by mechanical pressure, rolling, etc., become wet, introducing other permeability characteristics. If the skin is ruptured or taken off, the pea will also change its metabolism, e.g., carbon dioxide production increases 30% (Wager, 1957). The increase in carbon dioxide production, 11.2 μmol per gram, during the first hour cannot be explained by γ-aminobutyric acid production, which is only about 10% of that value. It has been reported that ethanol is produced by anaerobic peas (Wager, 1959), but there is still an excess of carbon dioxide produced.

The formation of γ-aminobutyric acid indicates that decarboxylation of L-glutamic acid is a main reaction in the removal of this acid. Further evidence for this theory is obtained in the tracer experiment, although part of the radioactivity was found in protein.

The higher activity of L-glutamic acid decarboxylase in damaged peas is explained by the fact that the enzyme is located mainly in the skin, where L-glutamic acid concentration is normally low. In damaged peas, however, there is a better enzyme-substrate contact. However, there must be a continuous supply of L-glutamic acid since the increase in γ-aminobutyric acid is not reflected in a corresponding decrease in L-glutamic acid concentration.

The experiments show that L-glutamine and probably protein, sugar, and starch are sources of L-glutamic acid, and secondarily

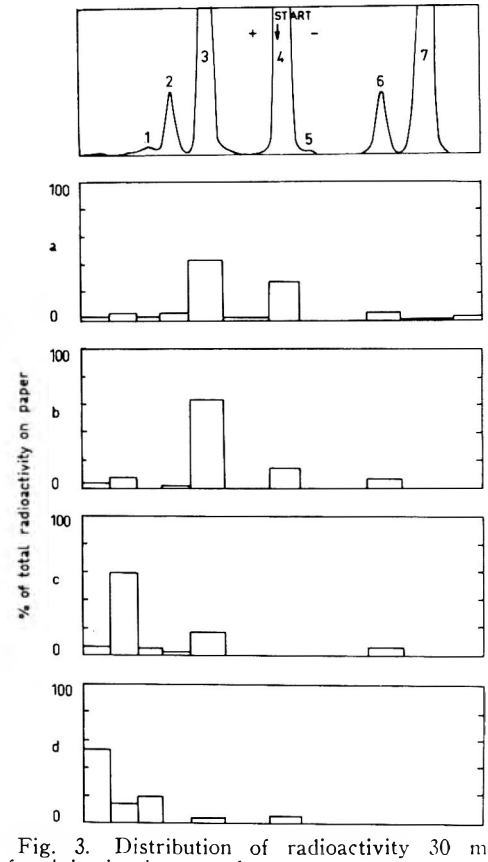


Fig. 3. Distribution of radioactivity 30 min after injection in peas of
a) L-Glutamine C^{14}, undamaged peas
b) L-Glutamine C^{14}, damaged peas
c) α-Ketoglutaric acid C^{14}, undamaged peas
d) L-Aspartic acid C^{14}, damaged peas.
The peaks are the same as in Fig. 2.

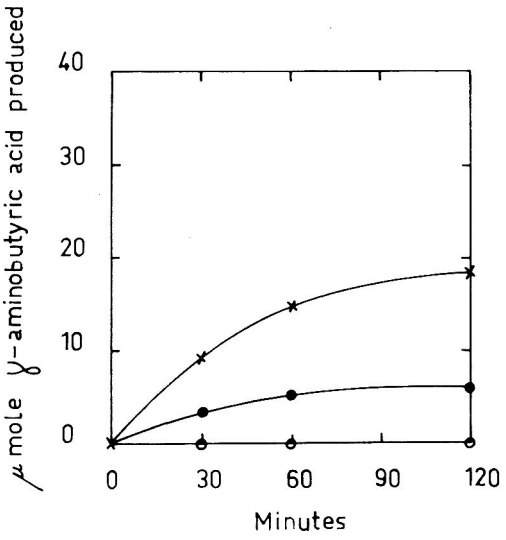


Fig. 4. L-Glutamic acid decarboxylase activity in washed tissue slices of peas. Temperature 37°C.
× = skin 1.40 g
● = outer part of cotyledon 1.93 g
○ = inner part of cotyledon 1.05 g. Volume of solution 10 ml.

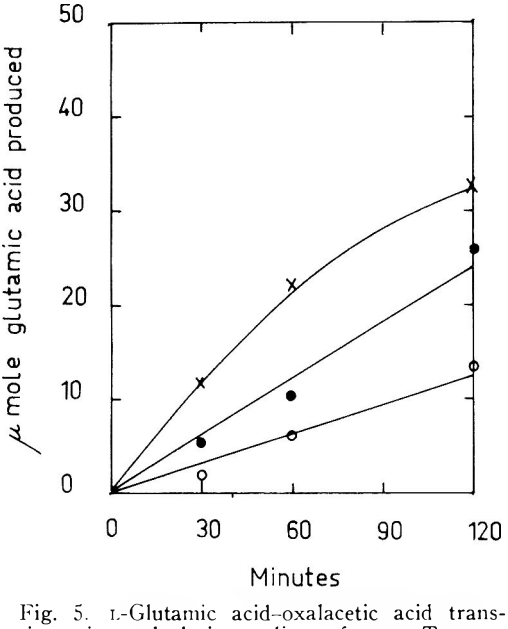


Fig. 5. L-Glutamic acid–oxalacetic acid transaminase in washed tissue slices of peas. Temperature 37°C.
× = skin 1.40 g
● = outer part of cotyledon 1.92 g
○ = inner part of cotyledon 0.99 g. Volume of solution 10 ml.

of γ-aminobutyric acid. By the action of L-glutaminase, proteolytic enzymes, and L-glutamic acid dehydrogenase, the concentration of L-glutamic acid is kept at a high value even though the decarboxylation rate is high, as in damaged peas.

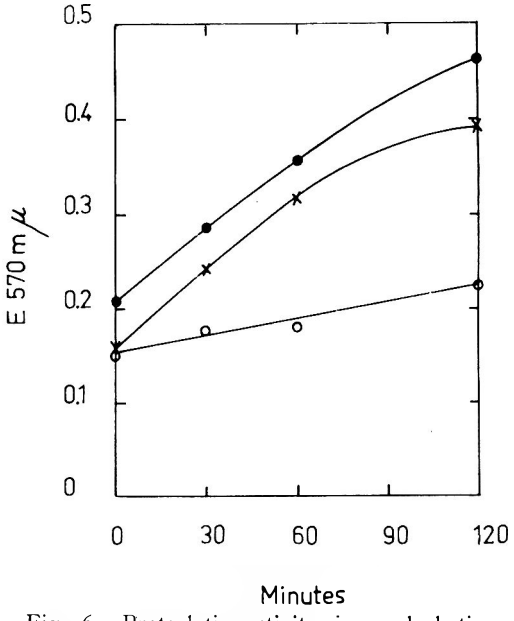


Fig. 6. Proteolytic activity in washed tissue slices of peas. Temperature 37°C.
● = outer part of cotyledon 4.60 g
× = skin 1.65 g
○ = inner part of cotyledon 2.01 g. Volume of solution 10 ml.

Transamination between L-aspartic acid and α-ketoglutaric acid also produces L-glutamic acid at the expense of L-aspartic acid. This reaction, indicated by the fact that radioactive L-aspartic acid is converted in oxalacetic acid, is interesting, because the enzyme L-glutamic acid–oxalacetic acid transaminase is located mainly in the outer parts of the pea, and the L-aspartic acid loss is explained by the better enzyme-substrate contact caused by damage. This reaction will go to completion, because the amount of L-aspartic acid, both free and bound, is not very high in peas and because the L-glutamic acid produced is decarboxylated by L-glutamic acid decarboxylase at the same site.

If the γ-aminobutyric acid content can be shown to represent a measure of the post-harvest time and treatment of peas, it will provide a new possibility of quality control.

ACKNOWLEDGMENT

The authors thank Mrs. A-M. Olsson for skillful technical assistance.

The work was supported by grants from the Swedish Technical Research Council.

REFERENCES

Auclair, J. L., and J. B. Maltais. 1952. Occurrence of γ-amino butyric acid in extracts of Pisum sativum (L) and in the honeydew of Myzus circumflexus (Buck). *Nature* **170**, 1114.

Danielsson, C.-E. 1951. Studies on a proteolytic enzyme from seeds of peas. *Acta Chem. Scand.* **5**, 791.

Danielsson, C.-E. 1952. A contribution to the study of the synthesis of the reserve proteins in ripening pea seeds. *Acta Chem. Scand.* **6**, 149.

Danielsson, C.-E., and H. Lis. 1952. Differences in the chemical composition of some pea proteins. *Acta Chem. Scand.* **6**, 139.

Gale, E. F. 1957. Determination of amino acids by use of bacterial amino acid decarboxylases. *In* "Methods of Biochemical Analysis." Vol. 4, 255. D. Glick (ed.).

Hac, L. R., M. L. Long, and M. J. Blish. 1949. The occurrence of free L-glutamic acid in various foods. *Food Technol.* **3**, 351.

Hanson, H. L., M. J. Brushway, and H. Lineweaver. 1960. Monosodium glutamate studies. I and II. *Food Technol.* **14**, 320.

Linko, P. 1960. Paper chromatogram patterns of free amino acids of wheats at various stages of deterioration. *Suomen Kemistilehti* B **33**, 144.

Mergentime, M., and E. H. Wiegand. 1946. Low temperature characteristics of a pea proteinase. *Fruit Prod. J.* **26**, 72.

Norton, K. B., D. K. Tressler, and L. D. Farkas. 1952. The use of monosodium glutamate in frozen foods. *Food Technol.* **6**, 405.

Schales, O., V. Mims, and S. S. Schales. 1946. Glutamic acid decarboxylase of higher plants. *Arch. Biochem.* **10**, 455 and **11**, 155.

Schuphan, W., and W. Postel. 1960. Biologische Wertigkeit des Erbseneiweisses im Spiegel der essentiellen Aminosäuren. *Z. Lebensm. Untersuch. u. -Forsch.* **113** 223.

Sjöström, L. B., and E. C. Crocker. 1948. The role of monosodium glutamate in the seasoning of certain vegetables. *Food Technol.* **2**, 317.

Soedigdo, R., and M. Gruber. 1960. Purification and some properties of a protease from pea seeds, Pisum sativum L, S.SP. Arvense A and G. *Biochim. et Biophys. Acta* **44**, 315.

Stumpf, P. K. 1951. Transaminases in higher plants. *Federation Proc.* **10**, 256.

van Duyne, F. O., V. R. Charles, M. C. Titus, and E. H. Wheeler. 1957. Effect of addition of monosodium glutamate on palatability of frozen vegetables. *Food Technol.* **11**, 250.

Wager, H. G. 1957. The effect of artificial wilting on the carbon dioxide production of developing pea seeds. *New Phytologist* **56**, 230.

Wager, H. G. 1959. The effect of artificial wilting on the production of ethanol by ripening pea seeds. *New Phytologist* **58**, 68.

Wager, H. G. 1962. The physiology of green peas in relation to storage. Paper B 2. 1. Part 2, First Intern. Congr. of Food Sci. and Technol., London 1962 (in press).

Biochemical Properties of Pork and Their Relationship to Quality[a,b] I. pH of Chilled, Aged and Cooked Muscle Tissue

R. G. KAUFFMAN,[c] Z. L. CARPENTER,[d] R. W. BRAY, AND W. G. HOEKSTRA

Departments of Meat and Animal Science and Biochemistry
University of Wisconsin, Madison

(Manuscript received May 10, 1963)

SUMMARY

Values for pH were obtained from the chilled, aged, and cooked loin and ham muscles originating from 439 pork carcasses. Muscle pH was compared to a number of objective and subjective measurements related to the qualitative and quantitative values of pork products. Increased muscle acidity was associated with pale, soft tissues, which yielded higher percentages of expressible juice. Chilled-muscle pH was associated with the pH of aged and cooked muscles, and there was also a positive relationship between the pH values of different muscles within each carcass at any stage of comparison. Dark, dry, firm muscle tissue exhibiting a relatively high pH, shrank less during curing and cooking and was more juicy and tender than pale, soft watery muscle tissue of low pH. Regardless of muscle acidity, the curing process raised all palatability ratings to a comparable and acceptable level; therefore it was concluded that the primary importance of a higher pork muscle pH, especially for hams, is its association with less shrinkage during processing.

INTRODUCTION

The pH of muscle tissue has been studied by a number of workers, and its change under varying conditions has been associated with the water-binding capacity of the muscle proteins, visual color appearance, and storage life of the product. Even though it has been assumed that muscle acidity is important in regulating shrinkage during processing and in influencing palatability, insufficient evidence is currently available. A study was therefore made of the relationship between muscle pH and some quantitative and qualitative traits of pork.

LITERATURE REVIEW

The ultimate pH of muscle post-mortem has been shown to be related to the amount of glycogen present at slaughter, and possibly with the activity of various enzymes, the concentrations of myoglobin and of certain inorganic ions, and the buffer capacity of the muscle (Hamm, 1953; Lawrie, 1955; Wismer-Pedersen and Briskey, 1961). It has also been implied (Briskey, 1958; Hall *et al.*, 1944; Wierbicki *et al.*, 1957) that as pH decreases to about 5.5, the isoelectric point of major pork muscle proteins is approached and these proteins consequently decrease in their electrostatic attraction for local water molecules, which results in increased amounts of "free," expressible water. In addition, water may be released by a reduction of forces dependent on capillary action, surface tension, and water dipole interaction. As water retention is lowered, protein molecules form a "closed" or "denatured" structure, which prevents deep penetration of light rays and therefore physically reflects greater quantities of the light, giving the muscle surface a lighter appearance. Furthermore, muscle rigidity lessens, which results in a soft, watery, pliable muscle.

Contradictory findings have been reported concerning the relationship of muscle acidity to the palatability characteristics of muscle tissue (Judge, 1958; Briskey, 1958; Stran-

---

[a] Published with the approval of the director of the Wisconsin Agricultural Experiment Station, Madison.

[b] This study was supported in part by research contract 12-25-010-577, U. S. Department of Agriculture, Marketing Research Division, and Oscar Mayer and Company, Madison, Wisconsin.

[c] Present address: Department of Animal Science, University of Illinois, Urbana.

[d] Present address: Department of Animal Husbandry, Texas A. & M. University, College Station, Texas. Contribution No. 372.

dine *et al.*, 1949). Some workers report an enhancement of tenderness accompanying a lower pH, whereas others imply that higher pH values are associated with more flavorful and tender pork; still others find no relationships between these variables. It has also been suggested that a dark, dry muscle of high pH shrinks less throughout processing but is less resistant to bacteriological spoilage.

EXPERIMENTAL METHODS

General procedure for this series of publications. The general procedures were described in an earlier report by Carpenter *et al.* (1963).

Specific procedures for the pH study. Data were obtained from 439 carcasses; however, because the carcasses representing animals of known history were analyzed separately from those from animals of unknown history, only the results of 233 carcasses from the latter group are discussed except when there were contradictory findings for the 206 carcasses of the known-history group. In addition, this paper includes a comparison of 199 hams (from the original 439), segregated into three groups according to color and firmness characteristics. The first group included soft, watery hams with light-colored muscles occurring adjacent to darker-colored muscles, causing a two-toned color; the second possessed "normal" grayish-pink muscles accompanied by moderately firm muscle structure; the final group were dark in color and extremely firm in structure. These groups represent the normal, and the two extremes in muscle color and firmness found in pork processing plants. All hams fitting these group specifications were included in this phase of the study.

At 24 hr post-mortem, a Beckman pocket pH meter was employed to obtain single pH values for the chilled longissimus dorsi muscle opposite the eighth rib, and for the gluteus medius and gluteus profundus muscles exposed in the surface of the ham butt. A Beckman Model G pH meter was used to measure the pH of the aged (5–8 days) muscles, which included the longissimus dorsi, semimembranosus, and biceps femoris. The pH of the same muscles was again measured after cooking and rechilling to 4°C. The semimembranosus and biceps femoris were not evaluated for pH 24 hr post-mortem, because it was considered important to leave the ham intact during the aging period.

Color and firmness scores were determined subjectively by visual appraisal and physical handling.

RESULTS AND DISCUSSION

As indicated in Table 1, the pH values of chilled, aged, and cooked muscles were generally related. Nevertheless it was shown that the pH value of the chilled longissimus dorsi was not a good indicator of its pH after the muscle had been aged or cooked. This can possibly be attributed to the small pH variation for a majority of the chilled loin muscles studied. One might suspect that processing would reduce pH variation among samples; however, a highly significant relationship ($r = .32$) existed between the pH of aged and cooked longissimus dorsi. The correlation coefficients were also higher for the pH of aged vs. cooked ham muscles than for the pH of chilled vs. aged muscles.

Table 1. Relationships between pH values of chilled, aged, and cooked pork.[a]

	Chilled muscles			Aged muscles			Cooked muscles
	Longissimus dorsi r[b]	Gluteus profundus r	Gluteus medius r	Longissimus dorsi r	Biceps femoris r	Semimembranosus r	Biceps femoris r
Chilled muscles							
Longissimus dorsi	....	....	.53**	....	....	....	....
Gluteus profundus	.28**	....	.55**	....	....	....	....
Aged muscles							
Longissimus dorsi	.13[c]	....	....	....	.38**	.35**	....
Biceps femoris	....	.40**	.46**	....	....	.83**	.51**
Semimembranosus	....	.41**	.52**	....	....	....	....
Cooked muscles							
Longissimus dorsi	.15[c]	....	....	.32**[c]	....	....	....
Biceps femoris	....	.35**	.35**	....	....	.51**	....
Semimembranosus	....	.29**	.37**	....	.52**	.51**	.78*

[a] N = 175.
[b] Simple correlations.
[c] N = 168.
*p ≤ .05.
**p ≤ .01.

Most of the comparisons between pH measurements of ham muscles were significantly related. Generally, the correlation coefficients were highest between the pH values of different muscles at similar stages of comparison. The relative pH of the chilled gluteus medius reflected that of the chilled gluteus profundus and longissimus dorsi. These data further suggest that different chilled, aged, and cooked ham muscles from the same carcass underwent similar acidity changes. About 25% of the variation in the cooked pH values was accounted for by the pH of aged samples, and 13% of the variation was associated with that of the chilled samples. This difference may be partially explained because the gluteus medius and gluteus profundus, which provided the chilled pH values, were not used for subsequent aged and cooked pH evaluations. The correlation coefficient between pH values of the chilled longissimus dorsi and the chilled gluteus medius was about double that between the chilled longissimus dorsi and the chilled gluteus profundus. This has been previously reported (Briskey, 1958) and suggests that the gluteus medius, like the longissimus dorsi, is most susceptible to the development of pale color and is primarily responsible for the variations in color of the ham butt surface. Even though the standard deviation for the gluteus profundus pH equalled that for the gluteus medius, its pH was sufficiently high to maintain a relatively dark color. The loin pH could therefore be used as an indicator of the gluteus medius pH and associated properties when only a cross-sectional surface of the longissimus dorsi was exposed in the intact carcass.

As anticipated from previous research (Briskey *et al.*, 1959; Lawrie, 1955; Wierbicki *et al.*, 1957), increased muscle acidity was associated with greater quantities of expressible juice. In addition, darker-colored muscles were firmer and exhibited higher pH values. Even though pH and firmness were related, it should be explained that muscles having higher pH values also contained more internal fat, which also may have contributed to greater firmness.

As shown in Table 2, muscle acidity and processing shrinkages were statistically related, but the magnitude of the correlation coefficients was relatively low. Muscles of high pH exhibited less shrinkage during curing and cooking, which can probably be explained by the decrease in total expressible juice with an increase in pH ($r = -.35$). Apparently the ability of muscles possessing a high pH to bind a greater percentage of water in the chilled state, is maintained throughout processing.

Table 2 also indicates a significant trend toward lower lean-cut yields from carcasses containing muscles having higher pH values. This would support the claim that, regardless of intramuscular fat content, muscles from leaner pork carcasses tend to have lower ultimate pH values and, consequently, softer, more watery muscles (Briskey, 1958).

Table 3 illustrates relationships of pH to palatability. Ham muscle acidity was not related to flavor. However, juiciness and tenderness were significantly enhanced when ham muscle pH values were higher. The significant relationships between subjective tenderness measurements and pH values of the ham muscles were supported by the highly significant negative correlations between pH and shear force. This pH-tenderness relationship is interesting in view of the fact that less acid muscle has a greater water-binding capacity. Also, expressible

Table 2. pH of three pork muscles vs. carcass leanness and shrinkage during processing.[a, b]

	% curing shrink	% cooking shrink	% lean cuts of carcass
pH values for:			
1) Chilled longissimus dorsi	....	−.23**	.09
2) Chilled gluteus medius	−.21**	−.38**	−.18**
3) Aged semimembranosus	−.28**	−.46**	−.17*
4) Cooked semimembranosus [c]	−.37**	−.39**	−.28**

[a] Simple correlations.
[b] N = 233.
[c] N = 175.
* $p \leqslant .05$.
** $p \leqslant .01$.

Table 3. pH vs. palatability factors for chilled, aged, and cooked pork.[a, b]

pH values for:	Juiciness	Flavor	Tenderness	
			Panel score	Shear force
1) Chilled longissimus dorsi	.13*(.18**)[c]	.04	.03	.11
2) Chilled gluteus medius	.20**	.01	.32**	—.22**
3) Aged semimembranosus	.27**	.06	.43**	—.28**
4) Cooked semimembranosus[d]	.22**	.02	.30**	—.20**

[a] Simple correlations.
[b] N = 233.
[c] Partial correlation holding % of intramuscular fat constant (applies only to coefficient in parentheses).
[d] N = 175.
*p ≤ .05.
**p ≤ .01.

juice, which varies inversely with pH, was associated with taste-panel scores for juiciness ($r = -.35$) and further substantiated the juiciness-pH relationship. The fact that lower pH values were associated with an increase in the production of free water, upholds the assumption that the end product originating from muscles with low pH would usually be drier after processing and thus reflect less acceptable juiciness ratings.

Results obtained for the 199 hams segregated according to color and firmness are shown in Table 4. The following general observations may be made: 1) over one-third

Table 4. Comparisons of quantitative and qualitative traits for 199 hams segregated according to color and firmness.

	I Light in color, soft and watery		II "Normal" in color moderately firm		III Dark in color, dry and firm	
	62[a]		94[a]		43[a]	
	35.5[b]		16.0[b]		23.3[b]	
	$\bar{x}$	sd	$\bar{x}$	sd	$\bar{x}$	sd
Physical and chemical properties						
% Intra- and intermuscular fat	7.12	1.87	7.72	2.48	8.83[g]	2.48
% expressible juice[c]	65.90	14.70	56.20[h]	15.40	52.60[h]	11.80
pH, chilled gluteus medius	5.71	.20	5.84[g]	.18	6.04[g]	.23
pH, aged semimembranosus	5.69	.23	5.84[g]	.27	6.06[g]	.30
pH, cooked semimembranosus[d, i]	6.13	.20	6.17	.22	6.34[g]	.29
Quantitative properties						
Chilled fresh weight (lb)	21.30	9.30	15.90[h]	6.50	16.80[h]	6.40
% curing shrink	4.50	5.50	3.30	5.50	0.50[g]	3.70
% cooking shrink, fresh hams[e]	40.60	2.00	39.20[g]	2.60	37.40[g]	2.80
% cooking shrink, cured hams[e, j]	11.40	1.90	11.20	2.00	10.90	2.00
Palatability ratings[f]						
Fresh ham scores	9.40	1.10	10.50[h]	1.20	10.90[h]	1.70
Cured ham scores[k]	12.40	0.70	12.70[h]	0.70	12.90[h]	0.80

[a] Total number of hams.
[b] % of hams originating from sow carcasses.
[c] Expressed as a percent of total moisture content of the sample.
[d] Some values missing; Group I $n = 39$, Group II $n = 69$, Group III $n = 24$.
[e] Includes both evaporation and drip losses.
[f] Based on a composite taste-panel score of separately determined and equally weighted values of tenderness, juiciness, and flavor; a composite score of 10.5 was considered acceptable.
[g] Mean is significantly different from other group means.
[h] Mean is significantly different from Group I mean only.
[i] All means are significantly different from means of pH of aged semimembranosus and chilled gluteus medius muscles.
[j] All means are significantly different from means of % cooking shrink of fresh hams.
[k] All means are significantly different from means of fresh-ham palatability scores.

of the soft and light-colored hams were produced from sow carcasses, yet they represented only about one-fourth of the total number of carcasses in the study; 2) the percent intra- and intermuscular fat was higher in firm and dark hams; 3) pH values were higher in dark-colored hams, regardless of stage of comparison; 4) the cooked muscles possessed pH values about .35 unit higher than those of uncooked muscles; 5) percent expressible juice was lower in dark, firm hams; 6) curing losses were about 4% (absolute) lower for dark, firm hams than for soft, light-colored hams; 7) fresh hams shrank about 3½ times as much as cured hams during cooking; 8) cooking shrink was less for dark and firm fresh hams, but little difference existed in cooking shrink among cured hams; 9) dark and firm hams received more desirable palatability ratings than did soft, watery hams; and 10) regardless of raw muscle color or firmness, cured hams were more acceptable in palatability than were fresh hams. It should be noted (data not presented) that when the variations of intra- and intermuscular fat and carcass weight were held constant, the magnitude and significance of the differences reported either did not change or were increased.

Even though a higher muscle pH was associated with more desirable juiciness and tenderness traits of fresh ham muscles, and to a less extent with their cured counterparts, in this study the curing process raised these quality attributes to an acceptable level of palatability, regardless of raw muscle classification. Therefore, the importance of pH level in the pork ham musculature was established through its association with shrinkages throughout processing. The pH-protein water-binding phenomenon was probably responsible for this relationship, as suggested in earlier research (Hamm, 1953).

REFERENCES

Briskey, E. J. 1958. The chemical and physical characteristics of certain pork ham muscles as associated with various physiological stress factors. Ph.D. thesis. University of Wisconsin Library, Madison.

Briskey, E. J., R. W. Bray, W. G. Hoekstra, P. H. Phillips, and R. H. Grummer. 1959. The chemical and physical characteristics of various pork ham muscle classes. *J. Animal Sci.* **18**, 146.

Carpenter, Z. L., R. G. Kauffman, R. W. Bray, and K. G. Weckel. 1963. Factors influencing quality in pork. B. Commercially cured bacon. *J. Food Sci.* **28**, 578.

Hall, J. L., C. E. Latschar, and D. L. Mackintosh, 1944. Characteristics of dark cutting beef. Survey and preliminary investigation. Kansas Agr. Expt. Sta. Tech. Bull. 58, Part IV.

Hamm, R. 1953. The water-binding capacity of meat in its economical importance. *Deut. Lebensm. Rundschau* **49**, 153.

Judge, M. D. 1958. Physical, chemical, and organoleptic relationships in fresh pork. M.S. thesis. Ohio State University Library, Columbus.

Lawrie, R. A. 1955. Residual glycogen at high ultimate pH in horse muscle. *Biochim. et Biophys. Acta* **17**, 282.

Strandine, E. J., C. H. Koonz, and J. M. Ramsbottom, 1949. A study of variations in muscles of beef and chicken. *J. Animal Sci.* **8**, 483.

Wierbicki, E., L. E. Kunkle, and F. E. Deatherage. 1957. Changes in the water-holding capacity and cationic shifts during the heating and freezing and thawing of meat as revealed by simple centrifugal method for measuring shrinkage. *Food Technol.* **11**, 69.

Wismer-Pedersen, J., and E. J. Briskey. 1961. Rate of anaerobic glycolysis versus structure in pork muscle. *Nature* **189**, 318.

Biochemical Properties of Pork and Their Relationship to Quality[a,b] II. Intramuscular Fat

R. G. KAUFFMAN,[c] Z. L. CARPENTER,[d] R. W. BRAY, AND W. G. HOEKSTRA

Departments of Meat and Animal Science and Biochemistry
University of Wisconsin, Madison

(Manuscript received May 10, 1963)

SUMMARY

Pork loin and ham muscles from a total of 439 carcasses were analyzed for total intramuscular fat, and the results were compared to certain qualitative and quantitative carcass traits. Greater quantities of fat were associated with higher flavor, tenderness, and, especially, higher juiciness ratings of the cooked fresh product. This increase in fat content was also accompanied by lower curing and cooking shrinkages and greater carcass fatness.

The curing process elevated palatability scores and lowered cooking shrinkages to a level that minimized the effect of intramuscular fat. It is therefore suggested that recognition of the importance of intramuscular fat be essentially confined to fresh pork products subjected to dry or semi-dry methods of cookery.

INTRODUCTION

Knowledge about animal fat is increasing rapidly, yet many features of its metabolism and function have not been clearly defined. Fat deposited subcutaneously, abdominally, intermuscularly, intramuscularly, intracellularly, and intravenously exists in a dynamic equilibrium. Intramuscular fat is of particular interest to meat scientists because there is evidence that it enhances the palatability traits of meat products.

This study was initiated to determine the relationships of intramuscular fat content to the qualitative and quantitative characteristics of fresh and cured pork. Furthermore, an attempt was made to determine the approximate amount of intramuscular fat necessary for acceptable palatability.

REVIEW OF LITERATURE

While researchers have only recently studied the significance of intramuscular fat in pork, its relationship to beef quality has been studied extensively. Cover and Hostetler (1960) and Cover *et al.* (1958) reported that the amount of intramuscular fat in beef was not consistently related to measures of tenderness, and therefore was not a good criterion for deciding between moist and dry methods of cooking. Hankins and Ellis (1939) and Wellington and Stouffer (1959) made similar findings. It was their conclusion that palatability is associated more closely with heredity and method of cookery than with intramuscular fat. From research involving over 300 pork loins, Judge (1958) concluded that intramuscular fat did not enhance tenderness. In contrast, other workers (Backus, 1958; Kropf and Graf, 1959; Palmer *et al.*, 1958; Simone *et al.*, 1958) showed that intramuscular fat in beef was related positively to the various palatability factors. In studying pork, Batcher and Dawson (1960) reported that the variation of intramuscular fat content accounted for about 65% of the variation in tenderness and juiciness; however, later research at the same experiment station (Batcher *et al.*, 1962) refuted previous find-

[a] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station, Madison.

[b] This study was supported in part by research contract 12-25-010-577, U. S. Department of Agriculture, Marketing Research Division, and Oscar Mayer and Company, Madison, Wisconsin.

[c] Present address: Department of Animal Science, University of Illinois, Urbana.

[d] Present address: Department of Animal Husbandry, Texas A. & M. College, College Station, Texas. Contribution No. 373.

ings by demonstrating that intramuscular fat was not responsible for the improved palatability of 283 pork roasts originating from 32 pork carcasses. Saffle and Bratzler (1959) reported a positive relationship between amounts of intramuscular fat and improved quality in pork. Even though Judge's (1958) findings indicated little significance for tenderness and flavor, they did show that intramuscular fat content was significantly related to juiciness ($r = .40$).

At present, little information exists relating intramuscular fat to such measurements as over-all carcass leanness and processing shrinkages. However, Saffle and Bratzler (1959) indicated that bacon curing shrinkages decreased with an increase in carcass fatness. In addition, defrosting drip losses in loin chops decreased significantly as fatness increased. Furthermore, no effect of carcass fatness was found on cooking shrink regardless of the cut of pork (except bacon) tested or the method of cooking employed.

EXPERIMENTAL METHODS

General procedures for this series of publications. The general procedures were described in an earlier report (Carpenter, 1963).

Scientific procedures for the intramuscular-fat study. Representative samples from the longissimus dorsi muscle opposite the 9th to 13th ribs and a set of eight composite muscles from a one-inch-thick center-cut ham slice were semi-frozen, ground three times in a food grinder, and stored at −20°C. Duplicate 3-g portions from each sample were dried 24 hr at 105°C for moisture analysis. Total fat from the dry samples was then extracted with diethyl ether for 24 hr by a Soxhlet extraction apparatus. Weight loss due to extraction was designated as total lipid content, and was expressed on both fresh-weight and moisture-free bases. The reported lipid values for the ham samples include, in addition to intramuscular fat, the portion of the intermuscular fat that was not removed prior to grinding.

Data are presented for the 233 unknown-history carcasses, which were selected according to carcass weight and degree of intramuscular fat in the loin. When results from the 206 known-history carcasses differed from those of the above group, the conflicting values were included in Table 1.

RESULTS AND DISCUSSION

Fig. 1 and Table 1 illustrate the the relationships between quantity of intramuscular fat and qualitative and quantitative considerations. As indicated by the partial correlation coefficients (Table 1), percent intramuscular fat was highly and directly associated with the three palatability characteristics of flavor, juiciness, and tenderness for the fresh loins and hams. Fat variation accounted for 50% of the variation in juiciness and, to a lesser extent, for variations in tenderness and flavor in the fresh loin samples. The coefficients were of a lower magnitude for the hams. The variation in internal fat of cured hams accounted for about 16% of the variation in juiciness score; however, the correlation coefficients for tenderness and flavor were somewhat lower, and also differed between the known- and unknown-history groups. These differences for hams may be partly due to the effects of the curing process and to the fact that a portion of the fat was intermuscular fat, which probably had little influence on the palatability of the muscles tested.

It is apparent from Table 1 and Fig. 1 that carcasses with higher lean yields possessed lower quantities of intramuscular fat. This association was anticipated, because increased intramuscular fat reflects to some extent a general increase in total carcass fat.

It was of particular interest to note that increased intramuscular fat content was, in some comparisons, related to lower curing and cooking shrinkages. Some disagreement prevails between the known- and unknown-history groups and between fresh and cured products; however, it was concluded that increased fat did not increase shrinkages, and may in fact have decreased them. For the cured hams, no significant relationship was observed between intramuscular fat and cooking shrinkage. This would suggest that the curing process may increase the water-holding capacity of the muscle proteins to such an extent that the effect of fat becomes insignificant. When the ham muscles contained greater quantities of fat, shrinkage during the curing process was significantly lower. Perhaps one might conclude that the water in meat products is more easily lost than is the internal fat that is surrounded by connective tissue and which is insoluble in brine. Consequently, muscle tissue con-

Table 1. Partial correlation coefficients of intramuscular fat content of loin and ham muscles with other qualitative and quantitative traits.[a]

	% intramuscular fat of:		
		Composite of 8 ham muscles	
	Longissimus dorsi	Fresh	Cured
Palatability traits			
Flavor score	.38**	.17*(.31**)[b]	.14*
Juiciness score	.70**	.51**	.40**
Tenderness score	.44**	.27**(.12)[b]	.27**(.08)[b]
Shear force (cooked)	−.35**	−.12	−.19**(−.13)[b]
Quantitative traits			
% carcass lean cuts	−.37**	−.38**	.....
% cooking shrink			
Fresh	−.04(−.23**)[b]	−.24**	.....
Cured	.....	.....	−.04
% curing shrink	.....	.....	−.24**

[a] Carcass weight statistically held constant. Data from 233 carcasses are represented.
[b] Known-history-carcass coefficients that differ with those of the unknown-history carcasses.
*$p \leqslant .05$.
**$p \leqslant .01$.

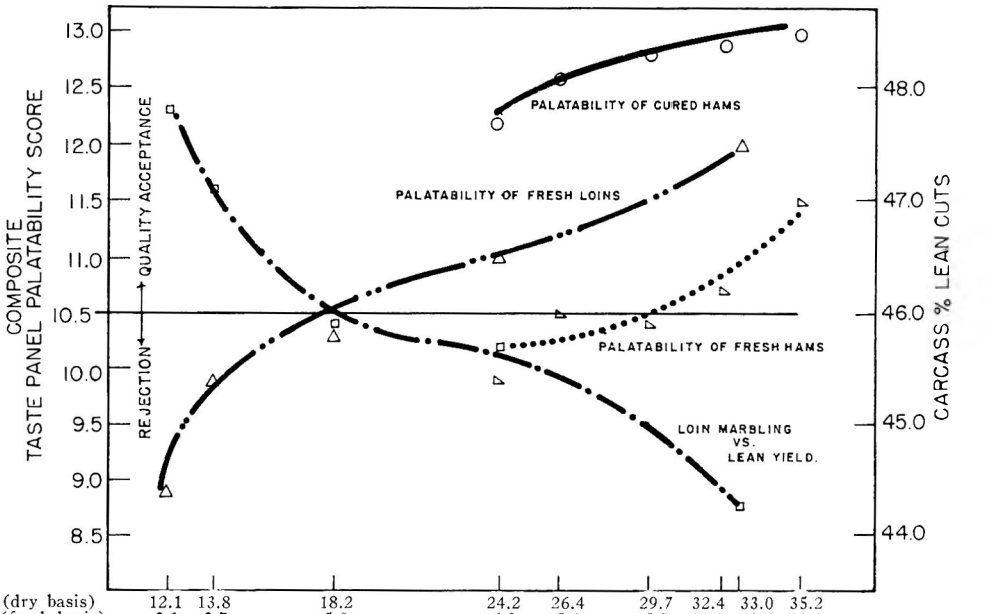
taining more fat and proportionately less water would be subject to relatively less shrinkage when subjected to curing. It is assumed that the losses of the moisture added during the curing process are equal for ham muscles possessing either high or low levels of fat.

The variance between the results of known- and unknown-history carcasses may have resulted from the lower content of intramuscular fat in the known-history group, especially for comparisons that gave correlations coefficients low in magnitude.

Fig. 1 illustrates the relationships of intramuscular fat to a composite palatability rating consisting of equally weighted values of panel scores of flavor, juiciness, and tenderness, and to a measure of carcass leanness. An "acceptance-rejection" line was established on the basis that the average composite palatability score for the three organoleptic traits on the hedonic scale was 10.5 and through the assumption that this level differentiated between acceptable and less desirable pork. This was an attempt to establish a demarcation point to provide for postulations concerning the data. Assuming that the line was realistic, it would appear that about 18–20% intramuscular fat, on a moisture-free basis (subjective score no. 3 = modest amounts of marbling), was necessary for acceptable palatability of fresh pork chops prepared by dry or semi-dry methods of cookery. Furthermore, a combination of about 30% inter- and intramuscular fat would be needed for braised fresh ham slices; however, the cured-ham palatability was acceptable regardless of fat content. When least significant differences were determined, the results indicated that differences existed in composite palatability scores for the various levels (marbling scores) of intramuscular fat in fresh pork, but in the ham most of these differences were masked by the curing process.

As previously noted in Table 1, there is a negative relationship between carcass leanness and percent intramuscular fat; this association is again demonstrated in Fig. 1. The study indicated that a lowering of lean-cut yields could be anticipated if palatability was to be maintained or improved. On the basis of present economic conditions, leanness is more important than the palatability characteristics of pork. Therefore it would be fatuous to suggest that intramuscular fat should be increased at the expense of leanness. Since intramuscular fat has not been included in a selection index for swine, little is known of its heritability. However, there is some evidence that leanness and intramuscular fat traits are not necessarily inherited in an antagonistic relationship and

Fig. 1. Relationships of intramuscular[a] fat to palatability and leanness.[b]



% fat (dry basis)	12.1	13.8	18.2	24.2	26.4	29.7	32.4	33.0	35.2
% fat (fresh basis)	3.1	3.7	5.0	6.9	8.1	8.7	10.0	10.4	11.1
Loin marbling score[c]	1	2	3	4(1)	(2)	(3)	(4)	5	(5)
Mean carcass wt. (lb)	195	189	196	189	189	196	189	190	190
n	46	47	50	47	47	50	47	43	43

[a] Also includes intermuscular fat for hams.
[b] 233 unknown history carcasses.
[c] Numbers in parentheses refer to loin marbling scores for carcasses yielding the corresponding fat contents of the hams.

may therefore be used together as selection tools for swine improvement.

This research suggests that the presence of some intramuscular fat is important to maintain acceptable palatability, but it does not explain how intramuscular fat may affect eating quality. Nonetheless, the authors postulate that intramuscular fat may serve as a lubricant between the muscle fibers and consequently improves juiciness. As juiciness is enhanced, flavor and tenderness may also be improved directly, or they may be rated higher psychologically solely because the product is more juicy. Although desirable flavor in pork may be partially the result of an increase or change in the nature of water-soluble constituents, the presence of rendered fat undoubtedly plays some part in the development of flavor. In addition, it is possible that the separation of muscle fibers and groups of muscle fibers by increased deposition of intramuscular fat may mechanically improve tenderness simply by reducing the concentration of connective tissue and by providing for a greater dispersion of degraded collagen (American Meat Institute Foundation, 1960).

REFERENCES

American Meat Institute Foundation. 1960. The science of meat and meat products. W. H. Freeman and Co., San Francisco and London, p. 29.

Backus, W. R. 1958. Specific gravity of the longissimus dorsi muscle from the 9–10–11 rib section as an objective measure of beef carcass eating quality. M.S. thesis. University of Tennessee Library, Knoxville.

Batcher, O. M., and E. H. Dawson. 1960. Consumer quality of selected muscles of raw and cooked pork. *Food Technol.* **14**, 69.

Batcher, O. M., E. H. Dawson, G. L. Gilpin, and J. N. Eisen. 1962. Good eating quality in lean pork roasts. Agr. Res., U.S.D.A., July.

Carpenter, Z. L., R. G. Kauffman, R. W. Bray, and K. G. Weckel. 1963. Factors influencing quality in pork. B. Commercially cured bacon. *J. Food Sci.* **28**, 578.

Cover, S., and R. L. Hostetler. 1960. An examination of some theories about beef tenderness by using new methods. Texas Agr. Expt. Sta. Bull. 947.

Cover, S., G. T. King, and O. D. Butler. 1958. Effect of carcass grades and fatness on tenderness of meat from steers of known history. Texas Agr. Expt. Sta. Bull. 889.

Hankins, O. G., and N. R. Ellis. 1939. Fat in relation to quantity and quality factors of meat animal carcasses. *Proc. Am. Soc. Animal Prod.* **314**.

Judge, M. D. 1958. Physical, chemical, and organoleptic relationships in fresh pork. M.S. thesis. Ohio State University Library, Columbus.

Kropf, D. H., and R. L. Graf. 1959. Interrelationships of subjective, chemical, and sensory evaluations of beef quality. *Food Technol.* **13**, 492.

Palmer, A. Z., J. W. Carpenter, R. L. Alsmeyer, H. L. Chapman, and W. G. Kirk. 1958. Simple correlations between carcass grade, marbling, ether extract of loin eye and beef tenderness. *J. Animal Sci.* **17**, 1153.

Saffle, R. L., and L. J. Bratzler. 1959. The effect of fatness on some processing and palatability characteristics of pork carcasses. *Food* Technol. **13**, 236.

Simone, M., F. Carroll, and M. T. Clegg. 1958. Effect of degree of finish on differences in quality factors of beef. *Food Research* **23**, 32.

Wellington, G. H., and J. R. Stouffer. 1959. Beef marbling. Its estimation and influence on tenderness and juiciness. Cornell Univ. Agr. Expt. Sta. Bull. 941.

Biochemical Properties of Pork and Their Relationship to Quality[a,b] III. Degree of Saturation and Moisture Content of Subcutaneous Fat

R. G. KAUFFMAN,[c] Z. L. CARPENTER,[d] R. W. BRAY, AND W. G. HOEKSTRA

Departments of Meat and Animal Science and Biochemistry
University of Wisconsin, Madison

(Manuscript received May 10, 1963)

SUMMARY

Degree of unsaturation and the moisture content of subcutaneous fatty tissue were compared to certain quantitative and qualitative traits of 247 pork carcasses. Degree of unsaturation and percent moisture of this tissue decreased as the fat content of the young animal increased; with mature packer sows, however, this pattern was not evident. Subcutaneous fat depots containing a greater amount of moisture and a relatively higher content of unsaturated fatty acids as determined by iodine number, were related to softer and leaner pork carcasses and were associated with less favorable palatability characteristics. Because lean, less firm carcasses possessed less intramuscular fat and therefore yielded pork cuts that generally had less desirable palatability, the subcutaneous fat characteristics may be associated only indirectly with quality attributes. Neither extent of unsaturation nor moisture content of the subcutaneous fatty tissue was related to the odor, as measured subjectively, of heated samples of fat.

INTRODUCTION AND REVIEW OF LITERATURE

Researchers (Hankins and Ellis, 1926; Ellis and Isbell, 1926; Hankins *et al.*, 1928; Blumer *et al.*, 1957; Dahl, 1958) have clearly demonstrated that the character of the subcutaneous fat of pork can be altered by nutrition. When swine were fed diets high in unsaturated fatty acids, soft pork fat resulted. Sinclair (1931) reported that no parallelism existed between iodine number of dietary fat and that of tissue phospholipids; however, the diet did alter the unsaturation of the body neutral fats. Cole (1955) subjectively determined the firmness of 59 pork carcasses and found that fat firmness was not as important in indicating over-all carcass firmness as reported in earlier studies. His studies indicated that firmness and degree of fatness were related directly. When Cole compared fats originating from soft and firm carcasses, he found that peroxide numbers did not differ after six months of storage; that no differences resulted in curing or cooking shrinkages; that palatability ratings were not altered; and that no marked rancidity was detected in either fat source after 12 months of storage.

Pork fat is susceptible to change in its degree of unsaturation during the growing and fattening period of swine; however, some studies imply that the firmness of fat post-mortem may also be the result of other factors not thoroughly understood. With the present emphasis on an accelerated production of trim, muscular pork, the relative importance of firmness of fat in evaluating pork must be established. Soft carcasses are presently criticized seriously even though this "softness" may be due primarily to superior muscling and not to highly unsaturated fat. This research was conducted to more clearly define the associations between

---

[a] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station, Madison.

[b] This study was supported in part by research contract 12-25-010-577, U. S. Department of Agriculture, Marketing Research Division, and Oscar Mayer and Company, Madison, Wisconsin.

[c] Present address: Department of Animal Science, University of Illinois, Urbana.

[d] Present address: Department of Animal Husbandry, Texas A. & M. College, College Station, Texas. Contribution No. 374.

two chemical measures of fat firmness (iodine number and moisture content of the fat) and some quantitative and qualitative traits of fresh and cured pork.

EXPERIMENTAL METHODS

General procedures for this series of experiments. The general procedures were described in an earlier report by Carpenter *et al.* (1963).

Specific procedures for the study of subcutaneous fat. Fat samples from 137 carcasses, randomly selected from the unknown-history group, and from 110 carcasses selected from the known-history group provided the data; however, results with known-history samples are reported only when they vary from those of the unknown-history group.

Iodine numbers were determined by the Hanus method as described by the AOAC (1960). A 600-g fat sample (including both inner and outer layers as delineated by the aponeurosa) was removed from the midsection (opposite the 7th and 8th ribs) of the fatback. Randomly selected cores of fat ($\frac{1}{2} \times 2$ inches) were removed from the samples, and the lipids were extracted with diethyl ether. The ether was evaporated at 40°C in a vacuum oven, and duplicate 0.3-g portions of extracted lipids were analyzed for iodine number.

Moisture content was determined on 2-g duplicate portions dried 36 hr at 105°C in shallow aluminum drying pans. Loss of weight during the drying period was recorded as percent moisture although it was realized that a small portion of this loss could have been attributed to volatile constituents other than water.

Carcass odor was analyzed subjectively. A 200-g fat sample was removed from the flank area of the chilled pork carcass, stored in a plastic bag, and then tested for odor within 24 hr. The fat sample was diced into small pieces and thoroughly mixed to provide a 30-g portion for analysis. This portion was added to 100 ml of distilled water contained in a 250-ml Erlenmeyer flask which was subsequently stoppered and slowly heated to 100°C in a hot-water bath. Two experienced panel members subjectively evaluated (by smell) the detectable volatile components released by the hot water-fat solution. The samples were classified into one of the three general groups. Group 1 consisted of normal odors producing mild sensations considered acceptable and pleasing. The second group included samples that yielded especially strong, objectionable sensations, which were often described as resembling "barnyard" odors. The final group involved odors not as severe as those in the second group but slightly abnormal and of questionable acceptability.

The bellies from the carcasses were subjectively evaluated for firmness and placed in one of five groups. The fluctuation of the 24-hr chilling temperatures and the fact that heavier carcasses failed to "chill out" as thoroughly as lighter ones undoubtedly affected the subjective firmness appraisal; however, the wide variation in firmness and the restriction to a five-group classification allowed for a reasonably accurate separation into firmness groups for statistical comparison.

RESULTS AND DISCUSSION

Data in the first two tables substantiate some of the relationships established by the previous research. When carcasses contained more total fat, the subcutaneous fat was more highly saturated and contained a lower percentage of moisture.

Even though the partial correlation coefficient for the known-history group (Table 1)

Table 1. Relationships between carcass traits and subcutaneous fat characteristics of 137 unknown-history pork carcasses.

Carcass traits	Iodine number $r_{12.345}$ [a]	% moisture of fat $r_{13.245}$ [a]	% variation of each trait accounted for by iodine number and % moisture of fat $R^2_{1.23}$ [a]
Subjective belly firmness score [c]	−44**	−.11 (−.38**)	32 (46)
Fatback thickness (inches)	−.28** (−.18*) [b]	−.38**	29 (52)
% Moisture—loin	.05 (.21*)	.19* (.30**)	17 (34)
% Moisture—ham	.19* (.00)	.15 (.30**)	16 (35)
Carcass weight (lb) [d]	.05 (.48**)	−.29** (−.45**)	9 (43)

* $p \leq .05$.
** $p \leq .01$.
[a] Identification of variables. 1 = individual carcass trait; 2 = iodine number; 3 = % moisture of fat; 4 = % intramuscular fat of loin (fatback thickness for the belly firmness comparison); 5 = carcass weight.
[b] All values in parentheses refer to 110 known-history carcasses.
[c] A score of 1 = soft; a score of 5 = firm.
[d] Omit variable no. 5 in this series of comparisons.

Table 2. Comparisons between subcutaneous fat characteristics and carcass traits classified according to origin and type of carcass.

		Unknown history			Known history		
		Butchers	Sows	Total	Butchers	Sows	Total
		114[a]	23[a]	137[a]	82[a]	28[a]	110[a]
Carcass traits							
Weight (lb)	$\bar{X}$	163.1	297.9	185.7	150.2	314.2	192.0
	S_d	39.9	12.4	62.4	39.4	78.6	88.5
% lean cuts	$\bar{X}$	46.4	46.1	46.3	49.4	46.6	48.7
	S_d	4.0	3.2	3.9	3.3	4.3	3.8
Fatback thickness (in.)	$\bar{X}$	1.76	2.18	1..83	1.45	2.18	1.64
	S_d	.37	.37	.40	.29	.48	.47
% intramuscular fat of loin[b]	$\bar{X}$	19.3	19.0	19.3	12.6	18.2	14.0
	S_d	8.8	9.3	8.9	5.3	6.5	6.1
Subcutaneous fat traits							
Iodine number	$\bar{X}$	61.7	63.1	61.9	61.7	65.5	62.7
	S_d	3.8	4.2	3.9	4.3	3.0	4.3
	r_{12}[c]	−.21*	.01	−.00	.09	.10	.35**
	r_{24}[c]	−.39**	−.29	−.37**	−.41**	.16	−.08
% moisture of fat	$\bar{X}$	7.1	6.6	7.0	8.0	5.7	7.4
	S_d	1.7	2.4	1.8	2.3	2.6	2.6
	r_{13}[c]	−.40**	−.21	−.29**	−.48**	−.36*	−.54**
	r_{34}[c]	−.21*	−.02	−.16	−.41**	−.29	−.47**

* $p \leq .05$.
** $p \leq .01$.
[a] Number of carcasses.
[b] Expressed on a moisture-free basis.
[c] Identification of variables: 1 = carcass weight; 2 = iodine number; 3 = % moisture of fat; 4 = % intramuscular fat of loin.

indicated that iodine number increased with an increase in weight, the simple correlation for the butcher carcasses of this group (Table 2) suggests that there was little relationship between these two variables. The partial analysis between carcass fatness and unsaturation simply implied that degree of unsaturation decreased with an increase in fattening rather than with carcass weight *per se*. Table 2 further illustrates this phenomenon. Correlations for the butcher groups indicated that the iodine number decreased with an increase in intramuscular fat deposition; however, this relationship was questionable for the heavier packer sow groups. In addition, the simple correlations showed that percent moisture in the fatty tissue also decreased with an increase in intramuscular fat content for butcher carcasses but not for the mature packer sows. These findings suggest that the biochemical nature of the fat depots from a mature

Table 3. Relationships between processing traits and subcutaneous fat characteristics of 137 unknown-history pork carcasses.

Processing traits	Iodine number $r_{12.345}$[a]	% moisture of fat $r_{13.245}$[a]	% variation of each trait accounted for by iodine number and % moisture of fat $R^2_{1.23}$[a]
% lean cuts of total carcass	.31**	.47**	39
% curing shrink of ham	−.04	.11	4 (19)
% cooking shrink of fresh loins	.23** (.06)[b]	−.04 (.28**)	5 (21)
% cooking shrink of fresh hams	.14	.30**	14

* $p \leq .05$.
** $p \leq .01$.
[a] Identification of variables. 1 = individual processing traits; 2 = iodine number; 3 = % moisture of fat; 4 = % intramuscular fat of loin; 5 = carcass weight.
[b] All values in parentheses refer to 110 known-history carcasses.

animal, regardless of degree of fatness, may be different from that of a young growing animal; however, the data do not explain this observed difference.

As anticipated, more highly saturated fats containing a minimum of moisture were physically firmer. Combined variation of the iodine number and water content of the fatty tissue statistically accounted for 32–46% of the changes in firmness. When iodine number was compared to the moisture content of the fatty tissue, no statistical relationship existed. This suggests that the two variables exerted their effect on firmness independently. Moisture content of the muscle was significantly correlated with iodine number; however, when the variations of intramuscular fat, carcass weight, and percent moisture of the fat were statistically held constant (Table 1), the correlation coefficients either failed to be significant or were of such low magnitude that they were of little predictive value. Percent moisture in the muscle tissue was related to percent moisture in the fatty tissue, especially for the carcasses from the pigs selected according to age. This was an anticipated relationship because the moisture content of fatty tissue decreased in carcasses containing more total fat and because an increased quantity of intramuscular fat was accompanied by a decreased moisture content of the muscles.

Comparisons between subcutaneous fatty tissue characteristics and some processing traits are presented in Table 3. The more muscular carcasses appeared to be less firm and contained fat of higher iodine number and higher moisture content. Because of the low correlation coefficients between the fat characteristics and curing and cooking shrinkages, and the disagreements between known- and unknown-history analysis, no definite conclusions could be reached. There was a tendency for meat with firmer fat characteristics as measured by iodine number and water content to exhibit a lower processing shrinkage.

Table 4 shows the relationships between fatty-tissue characteristics and measures of quality. Firmer subcutaneous fat depots with a lower iodine number and less water content were associated with higher palat-

Table 4. Relations between qualitative traits and subcutaneous fat characteristics of 137 unknown-history pork loins.

Qualitative traits [c]	Iodine number of fatback		% moisture of fatback		% variation in each trait accounted for by iodine number and % moisture of fatback $R^2_{1,23}$ [a]
	r_{12} [a]	$r_{12.345}$ [a]	r_{13} [a]	$r_{13.245}$ [a]	
Flavor, panel score	−.40**	−.28**	−.22**	−.26**	18
Juiciness, panel score	−.43**	−.25** (−.39**)	−.18* (−.33**)	−.14 (−.21*)	20 (29)
Tenderness, panel score	−.43**	−.30** (−.17)	−.22**	−.23** (−.32**)	21
Tenderness, shear force (lb/in.^{2})	.30** (.52**) [b]	.17* (.44**)	.13	−.10	10 (29)
Subjective odor score of flank fat	.22** (.09)	.....	.17* (−.06)	.....	.....

* $p \leqslant .05$.
** $p \leqslant .01$.
[a] Identification of variables. 1 = individual qualitative traits; 2 = iodine number; 3 = % moisture of fat; 4 = % intramuscular fat of loin; 5 = carcass weight.
[b] All values in parentheses refer to 110 known-history carcasses.
[c] Panel score: 1 = undesirable; 6 = desirable.

ability ratings for fresh, baked loin chops. This may be explained by the observation that the more muscular carcasses, which were usually less desirable in palatability characteristics, also contained a softer fat (as defined chemically). Collectively, degree of saturation and moisture content of the fat accounted for 10–29% of the variation in the various organoleptic traits. Nonetheless, when the results of odor tests were compared to the subcutaneous fat traits, little relationship existed. Because of conflicting results between the two groups studied, there was doubt as to the importance played by fat saturation or moisture content of the fat in predicting undesirable odors of pork fat; however, it should be noted that a large majority of samples received an acceptable rating, and thus the odor variation was minimized. Even though the fat was not analyzed for degree of rancidity, it is assumed that softer fats throughout the carcass may be more susceptible to rancidity and might therefore have some influence on the flavor and odor of cooked pork.

The data reveal differences between the known- and unknown-history groups. The values usually agreed in direction of relationship; however, the coefficients were generally higher for the known-history group. One explanation may be the difference in muscling between the two groups. As illustrated in Table 2, the mean lean yield was 2.4% higher ($p \leq .001$), the fatback thickness was .19 inch less ($p \leq .001$), and intramuscular fat content of the loin was 5.3% less for the known-history group. These differences may reflect a different basic population with greater variability in the fatty tissue composition. Breeding and management practices were unknown for the other group, and it was therefore impossible to account for any effect that these factors may have had.

REFERENCES

AOAC. 1960. "Official Methods of Analysis." 9th ed. Assoc. Offic. Agr. Chemists. 361.

Blumer, T. N., E. R. Barrick, W. L. Brown, F. H. Smith, and W. W. G. Smart, Jr. 1957. Influence of changing the kind of fat in the diet at various weight intervals on carcass fat characteristics of swine. *J. Animal Sci.* **16**, 68.

Carpenter, Z. L., R. G. Kauffman, R. W. Bray, and K. G. Weckel. 1963. Factors influencing quality in pork. B. Commercially cured bacon. *J. Food Sci.* **28**, 578.

Cole, J. W. 1955. Effects of fat firmness and degree of finish on evaluation of pork carcasses. Proc. Eighth Recip. Meats Conf., 115.

Dahl, O. 1958. Influence of the basal diet on the quality of pig fat. I. *Acta Agr. Scand.* **8**, 106.

Ellis, N. R., and H. S. Isbell. 1926. Soft pork studies. II. The influence of the character of the ration upon the composition of the body fat of hogs. *J. Biol. Chem.* **69**, 219.

Hankins, O. G., and N. R. Ellis. 1926. Some results of soft-pork investigations. I. U.S. Dept. Agr. Bull. No. 1407.

Hankins, O. G., N. R. Ellis, and J. H. Zeller. 1928. Some results of soft-pork investigations. II. U. S. Dept. Agr. Bull. No. 1492.

Sinclair, R. G. 1931. The metabolism of the phospholipids. III. The comparative influence of various fats on the degree of unsaturation of the phospholipids and neutral fat in the tissues of the rat. *J. Biol. Chem.* **92**, 245.

Radiosensitization of Streptococcus faecalis and Escherichia coli[a]

MOHAMED A. NOAMAN, GERALD J. SILVERMAN, NORMAN S. DAVIS, AND SAMUEL A. GOLDBLITH

Department of Nutrition and Food Science
Massachusetts Institute of Technology
Cambridge, Massachusetts

(Manuscript received June 17, 1963)

SUMMARY

The radiosensitizing activity of a series of naphthalene derivatives against *Streptococcus faecalis* and *Escherichia coli* was examined to determine the essential molecular substituents and configurations required for effective sensitization when the compounds and organisms were irradiated with Co^{60} gamma rays. In general, compounds with hydroxyl or amino groups in the ortho and para positions, such as 1-amino-2-naphthol and 4-amino-1-naphthol, were particularly effective sensitizers for *S. faecalis* irradiated in air or in anoxia. *E. coli* was particularly sensitive to 4-amino-1-naphthol and 5-amino-1-naphthol when irradiated in anoxia, and less sensitive in air. Both organisms displayed marked sensitivity when irradiated with iodoacetic acid in air and in anoxia. It was determined that the bacteria and chemicals must be irradiated together for maximal radiosensitization to take place, for it was possible to almost eliminate radiosensitization by washing the cell-chemical suspension prior to irradiation.

INTRODUCTION

An increasing number of radiosensitizers are being reported, and various mechanisms are proposed to explain their ability to amplify radiolethality. These are *N*-ethylmaleimide, iodoacetic acid, and phenylmercuric acetate (Bridges, 1961, 1962a); iodoacetamide (Dean and Alexander, 1962); chloral hydrate (Burns *et al.*, 1963); and the naphthalene compounds structurally related to vitamin K_5 (El-Tabel Shehata, 1961; Silverman *et al.*, 1962). In a sense, the expanding number of compounds studied can be attributed to an increased awareness that experiments with radiosensitizers may contribute significantly toward explaining mechanisms involved in radiolethality.

This work was undertaken to compare the behavior of different classes of radiosensitizers and to attempt to elucidate certain aspects of the action of naphthalene derivatives in radiosensitization.

EXPERIMENTAL

Organisms. Strains of *Streptococcus faecalis* (ATCC 10C1) and *Escherichia coli* (ATCC 9637) were propagated and enumerated by techniques used in previous studies (El-Tabey Shehata, 1961; Silverman *et al.*, 1962).

Chemical compounds. The sources of the chemicals used were: Vitamin K_5 HCl (K_5, Parke Davis and Co.); 1-naphthol (1N), 1-naphthylamine HCl (1NA), 4-amino-1-naphthol HCl (4A1N), 1,4-naphthalenediol (4H1N), 1,4-diaminonaphthalene diHCl (1,4ANA), 5-amino-1-naphthol HCl (5A1N), 1-amino-2-naphthol HCl (1A2N), hydroquinone (HQ), *p*-phenylenediamine diHCl (PDA), *p*-aminophenol (PAP), from Distillation Products Inc.; 2-amino-1-naphthol HCl (2A1N), 2-amino-3-naphthol (2A3N), beta-thionaphthol (2S1N), 2,3-dihydroxynaphthalene (3H2N), 2,3-diaminonaphthalene (2,3ANA), from K and K Lab., Inc.; maleic acid, 2-methylnaphthalene (2MN), from Matheson, Coleman and Bell; 1,2-diaminonaphthalene (1,2ANA), from Aldrich Chemical Co.; *N*-ethylmaleimide (NEM), naphthoresorcinol (3H1N), from Schwarz Bioresearch, Inc.; malonic acid, from Calbiochem; *p*-chloromercuribenzoic acid (PCB), iodoacetic acid (IAA), from Mann Research Lab.; sorbic acid from Carbide and Carbon Chemicals Co.

[a] Contribution number 546 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts.

Buffer. Anhydrous sodium monobasic phosphate (9.2 g) was dissolved in 600 ml of distilled water and the pH adjusted to 7 with sodium hydroxide and diluted to a final volume of 1 L. Before use, the buffer was diluted further to a final concentration of 0.022*M*.

Sparging and irradiation. In most experiments, evaluation of radiolethality of suspensions of organisms and chemical was done in sealed ampoules, but for certain experiments (see Table 6) continuous sparging was found to be necessary during irradiation. This apparatus consisted of four 100-ml tubes, each containing 50 ml of either a cell suspension or chemical, into which a coarse fritted-glass sparger was inserted so as to reach almost to the bottom of the liquid. Air or nitrogen was sterilized by passage through sterile glass wool placed at the manifold inlet and discharged through a filtered outlet. Samples were withdrawn with a hypodermic syringe containing an 8-in. 18-ga. needle through a stopcock valve on the side of the tube. Results with continuous sparging were found to be more precise than the ampoule technique. The artifact due to trapping of microorganisms on the fritted-glass filter, noted by Ginsberg and Jagger (1962), did not occur with this apparatus.

Irradiation. Irradiation was conducted in a submerged pool-type Co^{60} source. The dose rate averaged 5250 r/min over the duration of these experiments.

Lysis. To determine whether or not radiosensitization might be due to lytic activity, 9 ml of *S. faecalis* cells prepared in the same manner as for irradiation were mixed with 3 ml of the appropriate concentrations of K_5, 4A1N, or distilled water. Percentage transmission at 450 mμ (Schaechter and Santomassino, 1962) was taken initially, every 30 min for 4 hr, and at 24 hr.

RESULTS

Radiosensitization. Table 1 compares the influence, during irradiation, of 16 naphthalene compounds against *S. faecalis* in air and nitrogen. Some essentially non-toxic compounds were found to have sufficient radiosensitizing activity to permit only 10% of the cells irradiated with chemical to form colonies, compared to the number of survivors (here considered 100%) when cells were irradiated without chemical. The dramatic activity of many of the compounds examined suggested that the designation "radiosensitizer" be restricted in this discussion to chemicals that gave survival

Table 1. The ability of various groups on naphthalene to increase radiolethality for *Streptococcus faecalis*.[a]

		Irradiated		Unirradiated	
		Relative survival fraction		Percent survival	
Code	Compound ($10^{-4}M$)	Air (35,000 rad)	Nitrogen (100,000 rad)	Air	Nitrogen
	Buffer, pH 7, 0.022*M*	1.0	1.0	100	100
K_5	Vitamin K_5	0.14	0.00088	89	69
2MN	2-methylnaphthalene	0.79	0.36	97	122
1N	1-naphthol	0.12	0.02	87	78
1NA	1-naphthylamine	0.0000038	0.16	60	80
4A1N	4-amino-1-naphthol	0.00012	0.000046	46	47
4H1N	1,4-naphthalenediol	0.083	0.0001	45	62
1,4ANA	1,4-diaminonaphthalene	0.019	0.00016	17	32
5A1N	5-amino-1-naphthol	1.78	0.0079	44	64
1A2N	1-amino-2-naphthol	0.0054	0.000026	76	23
1,2ANA	1,2-diaminonaphthalene	0.15	0.014	45	2
2A1N	2-amino-1-naphthol	0.27	0.0016	0.027	0.14
2S1N	beta-thionaphthol	0.95	1.26	91	75
3H2N	2,3-dihydroxynaphthalene	1.58	0.0013	93	111
2,3ANA	2,3-diaminonaphthalene	0.56	1.26	65	90
2A3N	2-amino-3-naphthol	0.17	0.73	73	83
3H1N	naphthoresorcinol	0.85	0.1	69	61

[a] Survival fraction (SF) $= \frac{\text{number of organisms after irradiation}}{\text{number of organisms before irradiation}} = \frac{N}{N_o}$; percent survival $= \frac{\text{number of organisms in chemical}}{\text{number of organisms in buffer}} \times 100$; relative survival fraction (RSF) $= \frac{\text{SF in the presence of chemical}}{\text{SF in buffer}}$; RSF of control samples $= 1$.

values of less than 1% of those for controls irradiated without chemical. It will be demonstrated herein that many chemicals sensitized cells so that much less than 1% of the irradiated population was viable. In agreement with previous work (El-Tabey Shehata, 1961; Silverman *et al.*, 1962, 1963), the effectiveness of amino- and hydroxynaphthalene compounds related to K_5 is more evident in nitrogen than in air. In addition to K_5, 4A1N, 1A2N, and 5A1N, compounds 4H1N, 1,4ANA, 1,2ANA, and 2A1N were also active in nitrogen and either inactive or less active in air. One compound, 1NA, behaved quite differently, being active in air but not in nitrogen. The location of the amino and hydroxyl groups on the naphthalene ring influences whether or not radiosensitization occurs. The activity of the K_5 molecule is due to the presence of the hydroxyl and amino groups; for if these groups are absent, as in 2MN, the activity is lost. A compound having amino or hydroxyl groups in the ortho and para positions, or even on the second benzene ring, as in 5A1N, can cause radiosensitization. Placing a sulfhydryl group adjacent to the hydroxyl in 2S1N appears to impart protective activity to 1N, a weak radiosensitizer. Although 3H2N was active in nitrogen, the corresponding naphthylamine, 2,3ANA, and the compound 2A3N were ineffective. Naphthoresorcinol (3H1N), which has two hydroxyl groups in a meta relationship, was also inactive. Four compounds, 4A1N, 4H1N, 1,4ANA, and 1A2N, not only were effective in nitrogen but also appreciably decreased the radiosurvival of *S. faecalis* in air. In general, it appears that compounds having either amino or hydroxyl groups in the para position are most effective sensitizers against *S. faecalis*. Their interchangeability is not evident in the 1- and 2-positions, since a greater radiolethality for *S. faecalis* was noted under anoxia in the presence of 1A2N than in the presence of 2A1N.

The radiolethal effect noted for K_5 is also demonstrated for *S. faecalis* by phenols and phenylamines (Table 2). These compounds not only are less effective sensitizers in anoxia than the corresponding naphthalenes but also differ in being inactive in air. Included for comparative purposes were two sensitizing compounds, NEM and IAA, reported by Bridges (1962a) for a *Pseudomonas* sp. Bridges had found that although NEM and IAA were ineffectual radiosensitizers in air, phenylmercuric acetate was active in air and in anoxia. In this study, at concentrations of $10^{-4}M$ they were comparable to, or greater sensitizers than, 4A1N or 1A2N. *p*-Chloromercuribenzoic acid (PMB), a sulfhydryl reagent, was also tested, and found to be an effective radiosensitizer in both air and nitrogen.

Table 2. Other compounds that increase the radiolethality of *Streptococcus faecalis*.

Code	Compound ($10^{-4}M$)	Irradiated: Relative survival fraction, Air (35,000 rad)	Irradiated: Relative survival fraction, Nitrogen (100,000 rad)	Unirradiated: Percent survival, Air	Unirradiated: Percent survival, Nitrogen
	Buffer, pH 7.0	1.0	1.0	100	100
HQ	Hydroquinone	0.5	0.00043	85	110
PAD	*p*-Phenylenediamine	1.21	0.00065	84	92
PAP	*p*-Aminophenol	0.15	0.00026	76	86
NEM	*N*-Ethylmaleimide	0.13	0.000079	85	97
PCM	*p*-Chloromercuribenzoic acid	0.00074	0.00000029	74	61
IAA	Iodoacetic acid	0.0000033	0.00000025	80	89
	Sorbic acid	1.78	0.07	62	82
	Maleic acid	1.22	0.98	88	81
	Malonic acid	0.57	0.26	124	93

Three additional compounds, sorbic, malonic, and maleic acids, were similarly examined. Of the three, sorbic acid was of interest; it appeared to be protective when the organisms were irradiated in air, and displayed a slight radiosensitizing activity in nitrogen.

Table 3 presents the results of experiments with *E. coli* and seven compounds that had increased the radiolethality of *S. faecalis*. Except for IAA, which showed a greater effect in air, the effects were most notable in nitrogen. *E. coli* differed from *S. faecalis* in being indifferent to the presence of 1NA in either air or nitrogen. Some results with *E. coli* are in agreement with a previous study by Bridges (1961).

Bridges investigated the effects of NEM on *Escherichia coli* B/r, *Staphylococcus aureus*, a *Pseudomonas* sp., and *Bacillus subtilis*. NEM activity at $10^{-3}M$ was more pronounced in anoxia against *E. coli* and *S. aureus* and only in anoxia

Table 3. The modification of radiosurvival of *Escherichia coli* in air and nitrogen by various chemicals.

Compound ($10^{-4}M$)	Irradiated: Relative survival fraction: Air (20,000 rad)	Irradiated: Relative survival fraction: Nitrogen (60,000 rad)	Unirradiated: Percent survival: Air	Unirradiated: Percent survival: Nitrogen
Buffer, pH 7.0	1.0	1.0	100	100
Vitamin K_5	0.73	0.0039	77	78
4-Amino-1-naphthol	0.16	0.00015	65	43
5-Amino-1-naphthol	0.16	0.00015	65	62
1-Naphthylamine	0.67	0.58	85	91
p-Aminophenol	0.73	0.013	90	94
N-Ethylmaleimide	0.95	0.05	84	100
Iodoacetic acid	0.000033	0.0027	87	130

in the case of the *Pseudomonas* sp. The radiosurvival of *B. subtilis* was not altered by NEM in either air or nitrogen.

Toxicity. Silverman *et al.* (1962) discussed certain aspects of the concepts of radiosensitization. The definitions usually state that a radiosensitizer should increase radiolethality without notable toxicity and manifest its sensitizing action only during irradiation (Bridges, 1962a; Eldjarn and Pihl, 1960). A certain amount of bactericidal action was encountered in this study (Tables 1, 2, 3), as in previous studies. Toxicity appeared to vary with the molecular configuration of the compound. The most toxic classes of compounds were those having amino or hydroxyl groups in the 1 and 2 positions of the naphthalene ring (1A2N, 1,2ANA, and 2A1N). Two of the most toxic (1,2ANA and 2A1N) were only moderate sensitizers. The other appreciably toxic compound was 1,4ANA—an effective sensitizer.

It was thought most pertinent to determine whether these compounds could be radiosensitizers in the absence of toxicity (Table 4). Although no data are presented for *E. coli*, both organisms reacted to these chemicals in a similar manner. It is interesting to note the low concentrations of chemical at which sensitization occurs. Wherever an increased toxicity was noted, the magnitude of radiosensitization was always greater.

Elimination of the bactericidal action may not, of course, prevent partial damage to a cell. This problem has been recognized and discussed by

Table 4. The effect of chemical concentration on the radiosurvival of *Streptococcus faecalis* in air and in nitrogen.

Compound	Molarity $\times 10^{-6}$	Irradiated: Relative survival fraction: Air (35,000 rad)	Irradiated: Relative survival fraction: Nitrogen (100,000 rad)	Unirradiated[a]: Percent survival: Air	Unirradiated[a]: Percent survival: Nitrogen
Buffer, pH 7.0		1.0	1.0	100	100
Vitamin K_5	100	0.14	0.00088	89	69
	50	0.29	0.0025	104	80
	10	0.48	0.014	118	91
4-Amino-1-naphthol	100	0.00012	0.000046	46	48
	50	0.011	0.00012	71	58
	10	0.24	0.0003	98	107
5-Amino-1-naphthol	100	.....	0.0025		72
	50	.....	0.015		89
	10	.....	0.036		100
	1	.....	0.6		111
1-Naphthylamine	100	0.000012	0.22	76	81
	50	0.0042	0.22	91	86
	10	0.39	0.21	95	99
Iodoacetic acid	100	0.0000042	0.0000003	86	82
	50	0.37	0.00083	96	103

[a] The initial concentration of organisms was 10^8 per ml in $0.022M$ buffer, pH 7.

Jacobs and Harris (1960) in their study of disinfectants. Exposure of cells to heat or chemicals, such as phenolic substances, can result in loss of essential cellular materials, and may make an organism susceptible to further lethal stresses (heat, etc.). Certain vitamin K derivatives can increase the thermal destruction of microbial spores (Michener *et al.*, 1959). In preliminary experiments to test this point *S. faecalis* was heated in the presence of buffer, and in concentrations of $10^{-4}M$ of K_5, and 4A1N. The thermal rate of destruction was considerably greater in air than in nitrogen, but the extent of thermal death was equally as great. Since these chemicals exert a greater radiosensitizing effect in nitrogen than in air, an additional factor(s) may be operative in the case of radiosensitization.

Lysis. Schaechter and Santomassino (1962) reported that cells in the logarithmic stage of growth are lysed in the presence of *p*-chloromercuribenzoic acid (PMB). They observed that cells in the stationary phase were immune to this lytic phenomenon. Although the cells used in the present experiments were stationary-phase cultures, spectrophotometric measurements for possible lysis were taken at intervals after the addition of K_5, 4A1N, and PMB, and no lysis was detected.

Chemical contact with cell. El-Tabey Shehata (1961) for K_5, and Bridges (1961) for NEM, noted that, for sensitization to occur, these chemicals had to be present during irradiation. This is also true for 4A1N and 1NA, and is also likely the case for the other sensitizing compounds discussed here (Tables 5, 6). The results in Table 5 substantiate the necessity for K_5 or 4A1N to be present during irradiation, and show that the influence of these chemicals is either minimized or eliminated by subsequent removal by washing prior to irradiation. A certain amount of residual activity was noted, but this was likely due to the additional washings involved. Experiments to verify this fact indicated that cells without chemical washed 4 times (the equivalent for this experiment) have an increased radiation sensitivity. This was verified when the greater radiosensitivity displayed upon readdition of the chemicals to washed suspensions of *S. faecalis* was compared to the values obtained by direct dilution. The results in Table 6 show that *S. faecalis* was not subject to the sensitizing action of K_5 or 4A1N unless both organism and chemical were irradiated together. Sensitization was not detected when the organism and the chemical were irradiated separately and then mixed in various irradiated and unirradiated combinations. This suggests that the lethal combination of either 4A1N or K_5 and

Table 5. The influence of washing suspension of various chemicals and *Streptococcus faecalis* before irradiation.

Compound ($10^{-4}M$)	Irradiated	
	Relative survival fraction	
	Air (35,000 rad)	Nitrogen (100,000 rad)
Buffer	1.0	1.0
Vitamin K_5		
a) control (washed cells and chemical)	.....	0.00038
b) cells washed two additional times after chemical was added	.....	0.23
c) cells and chemical diluted 1/1000 before irradiation [a]	.....	0.11
d) cells washed twice to remove the chemical and the chemical readded before irradiation	.....	0.000021
4-Amino-1-naphthol		
a) control (washed cells and chemical)	0.000045	0.000025
b) cells washed two additional times after chemical was added	0.065	0.015
c) cells and chemical diluted 1/1000 before irradiation [a]	.....	0.13
d) cells washed twice to remove chemical and the chemical readded before irradiation	.....	0.0000044
1-Naphthylamine		
a) control (washed cells and chemical)	0.0000045	.....
b) cells washed two additional times after chemical was added	0.73	

[a] Unlike the other suspensions, of 10^8 cells/ml, this suspension contained 10^5 cells/ml.

Table 6. The effectiveness of vitamin K_5 and 4-amino-1-naphthol (4A1N) on *Streptococcus faecalis* when mixed in various irradiated and unirradiated combinations in nitrogen.[a]

Compounds ($10^{-4}M$)	Irradiated (100,000 rad) Survival fraction
Irradiation of a mixture of chemical and organisms	
Buffer	0.04
4A1N	0.00000023
Vitamin K_5	0.0000032
Addition of unirradiated chemical to irradiated organisms	
4A1N	0.10
Vitamin K_5	0.22
Addition of irradiated chemicals to unirradiated organisms	
4A1N	1.06
Vitamin K_5	0.93
Addition of irradiated chemicals to irradiated organisms	
4A1N	0.081
Vitamin K_5	0.34

[a] Irradiation was conducted on the continuous sparging apparatus.

S. faecalis may be due to a short-lived activated molecule that is in intimate contact with the cell.

DISCUSSION

Of the hydroxyl-naphthol and phenolic compounds tested, those compounds possessing amino or hydroxyl groups in the para or ortho configuration possessed the greatest radiosensitizing activity. Compounds having hydroxyl or amino groups in the less reactive meta or 2,3-positions are inactive. Bachofer and Hartwig (1956) examined the effect of *o*-, *p*, and *m*-hydroxy- and amino-benzoic acids on the radiosurvival of coliphage T1. Ortho- and para-aminobenzoic and hydroxybenzoic acids gave a higher protective action than the meta compound, the aminobenzoic acids being more efficient than the hydroxylated acids. Irradiation by Bachofer and Hartwig was in air only, so that possible anoxic sensitization would not be detected.

A wide variety of compounds are known to be capable of combining with sulfhydryl groups, and those sulfhydryl reagents examined in this study (NEM, IAA, PMB) also displayed an ability to act as radiosensitizers.

Bridges (1962b) in studying mutation rates concluded that, if NEM is involved primarily with S—H groups, the initial lesion should occur on sulfhydryl-containing proteins associated with DNA instead of DNA itself. It is therefore possible that radiosensitization events may occur as specific targets that may not be nucleic acids. Alper (1961) reviewed the evidence against necessarily considering nucleic acids as the site of the primary lethal lesion in radiolethality. Dean and Alexander (1962) believe that iodcacetamide acts primarily in preventing restoration of the original physiological state of the cell surface and membrane. In a recent study, Silverman *et al.* (1963) demonstrated the neutralizing effects of the sulfhydryl moiety (cysteine and glutathione) and food constituents on radiosensitization by K_5, 4A1N, and 1A2N. Until further work is done to correlate the sulfhydryl binding capacities of those compounds examined in this study with their sensitizing activity, the possibility still exists that neutralization by sulfhydryl compounds may reflect an associated reaction and not the specific mechanism involved in radiosensitization by naphthalene compounds.

The responses of the organisms studied in this and other investigations indicate that a variety of responses are to be encountered in regard to anoxia, microbial species, and structure of the sensitizer. Certain bacteria produce compounds possessing vitamin K activity (Jacobsen and Dam, 1960); and naphthoquinone can also be microbial growth-promoting factors under anoxia (MaciasR, 1961). It is not likely that an organism's susceptibility is due to its vita-

min K content, since a wide variety of protective compounds are also present within a cell.

A number of structurally different classes of protectors exist which react with the energy of ionizing radiation in different manners. There are many postulates as to why protectors are effective, especially those pertaining to sulfhydryl compounds (Alper, 1961; Eldjarn and Pihl, 1960). It is not unreasonable to suppose that distinct classes of sensitizers may also exist, which can exert their synergistic action in a manner predicated by their structures.

The observations that anoxia is more conducive for sensitization, that certain naphthalene compounds are effective only if present during irradiation, and that sulfhydryl compounds and proteins tend to neutralize their activity, indicate that these compounds can interact with certain cell constituents and that the radiolethal action on a cell is governed by the cell's response to products formed during irradiation. Further confirmation will depend upon experiments that are expected to determine the extent of penetration of these compounds into a cell and their behavior during irradiation.

REFERENCES

Alper, T. 1961. Effects on subcellular units and free-living cells. *In* "Mechanisms in Radiobiology." (A. Errera and A. Forssberg, eds.) Vol. 1, pp. 353–412. Academic Press, New York.

Bachofer, C. S., and Q. L. Hartwig. 1956. Relation of structural configuration to protection against ionizing radiations. *Radiation Research* **5**, 528.

Bridges, B. A. 1961. The effect of *N*-ethylmaleimide on the radiation sensitivity of bacteria. *J. Gen. Microbiol.* **26**, 467.

Bridges, B. A. 1962a. The chemical sensitization of Pseudomonas species to ionizing radiation. *Radiation Research* **16**, 232.

Bridges, B. A. 1962b. Enhancement of radiation-induced mutation rate by *N*-ethylmaleimide. Abstr. in Second Intern. Congr. Radiation Research, p. 16, Harrogate, England.

Burns, J., R. F. Pittillo, and J. P. Garcia. 1963. Potentiation of radiation damage in microorganisms by chloral hydrate and related compounds. *Bacteriol. Proc. Am. Soc. Microbiol.* 63rd Ann. Meeting.

Dean, C. J., and P. Alexander. 1962. Radiosensitization of bacteria with iodoacetamide. Abstr. in Second Intern. Congr. Radiation Research, p. 191, Harrogate, England.

Eldjarn, L., and A. Pihl. 1960. Mechanisms of protective and sensitizing action. *In* "Mechanisms in Radiobiology." (M. Errera and A. Forssberg, eds.) Vol. II. Academic Press, New York.

El-Tabey Shehata, A. M. 1961. Effect of combined action of ionizing radiation and chemical preservatives on microorganisms. *Radiation Research* **15**, 78.

Ginsberg, D. M., and J. Jagger. 1962. Possible errors arising from the use of fritted-glass filters for bubbling of cell suspensions, especially in irradiation experiments. *J. Bacteriol.* **83**, 1361.

Jacobs, S. E., and N. D. Harris. 1960. The effect of environmental conditions on the viability and growth of bacteria damaged by phenols. *J. Appl. Bacteriol.* **23**, 294.

Jacobsen, B. K., and H. Dam. 1960. Vitamin K in bacteria. *Biochim. et Biophys. Acta.* **40**, 211.

MaciasR, R. M. 1961. Phthiocol and menadione as acetate-replacing factors for Lactobacillus delbrueckii. *J. Bacteriol.* **82**, 657.

Michener, H. D., P. A. Thompson, and J. C. Lewis. 1959. Search for substances which reduce the heat resistance of bacterial spores. *Appl. Microbiol.* **7**, 166.

Schaechter, M., and K. A. Santomassino. 1962. Lysis of Escherichia coli by sulfhydryl-binding reagents. *J. Bacteriol.* **84**, 318.

Silverman, G. J., A. M. El-Tabey Shehata, and S. A. Goldblith. 1962. The radiosensitivity of Escherichia coli and Streptococcus faecalis as influenced by vitamin K_5 and its analogs. *Radiation Research* **16**, 432.

Silverman, G. J., N. S. Davis, and S. A. Goldblith. 1963. Modification of radiolethality by vitamin K_5 and certain analogs in model systems and in foods. *Food Technol.* Submitted for publication.

Incidence of Putrefactive Anaerobic Spores in Meat[a]

K. H. STEINKRAUS[b] AND J. C. AYRES
Departments of Bacteriology and Dairy and Food Industry
Iowa State University, Ames, Iowa

(Manuscript received April 11, 1963)

SUMMARY

Study of the incidence of putrefactive anaerobic spores in fresh and cured pork trimmings and in canned pork luncheon meat from several commercial meat packing plants in Iowa indicated that the level of contamination was very low. The mode of putrefactive anaerobic spores in fresh pork trimmings was less than 0.18 per gram and less than 1 per gram in cured pork trimmings and canned pork luncheon meat. The maximum spore count found in any sample tested was 51 spores per gram. Spore counts on samples from different plants were similar.

Little work has been reported on the incidence of putrefactive anaerobic spores in meat. This is rather surprising since the higher the level of these organisms, the greater would be the possibility that *Clostridium botulinum* might be present. Harriman *et al.* (1948) indicated that there was an average load of 2–4 anaerobic spores per gram in fresh and cured pork trimmings and in pork luncheon meat. The highest count they obtained was 110 spores per gram of meat. No spores of *Clostridium botulinum* were found. Burke *et al.* (1950), in 43 determinations on the incidence of putrefactive anaerobic spores in fresh, cured, and heat-processed pork trimmings, found that the average load in each of the three types of samples was less than one spore per gram. Ayres and Adams (1953) later reported that, in a study of 36 matched sets of knuckle, inside round, and outside round of cutter and canner grade beef, the usual numbers of putrefactive spores growing anaerobically were 0.7–6.0 per 100 g of meat.

An investigation was made to extend earlier work on the incidence of putrefactive anaerobic spores in fresh, cured, and processed pork trimmings. Brief attention was given to the incidence of putrefactive anaerobic spores in fresh beef trimmings.

EXPERIMENTAL

Meat samples. Fresh pork trimmings were secured from four commercial meat packing plants in different sections of Iowa, hereinafter designated as plants A, B, C, and D. The samples, collected during a three-year period, represented production for all seasons of the year.

Fresh beef trimmings were purchased from a retail outlet handling meat from plant E. The beef was held 6–10 days in the store at 2.5±2°C, chuck and plate trimmings were then ground through a 5/16-inch meat plate.

Bacteriological media. Linden thioglycollate starch medium (Difco + 0.1% soluble starch) was used for all determinations of incidence of putrefactive anaerobic spores in meat. Final pH of the medium was 7.2. When a solid medium was desired for growing surface colonies of organisms or for anaerobic plates, 1.5% agar was added. Usually, 10-ml quantities of media were placed in each 18×150-mm test tube. When low

---

[a] Journal Paper J-4577 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project No. 1264; Center for Agricultural and Economic Development Cooperating.

This paper reports research undertaken in cooperation with the Armed Forces Food and Container Institute, U. S. Army Quartermaster Research and Engineering Center, and has been assigned number 2274 in the series of papers approved for publication. The views or conclusions are those of the authors, and are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

Subsequent to completion of this work, an excellent review article by H. Riemann, "Safe Heat Processing of Canned Cured Meats with Regard to Bacterial Spores," appeared in *Food Technology* 17(1), 39–49 (1963).

[b] Present address: Dept. of Food Science and Technology, Cornell University, Geneva, New York.

spore counts necessitated the use of large inocula of meat, 25-ml amounts of media were placed in 25×200-mm test tubes.

Preparation of spore suspensions. Linden thioglycollate starch medium with 1.5% agar added was used to grow organisms for production of spores. The medium was placed in tubes and allowed to solidify in slants, providing a large surface area. The anaerobic organisms were streaked on the surface of the medium, and the tubes were incubated 2 weeks at 37°C in an atmosphere of nitrogen. Sterile physiological saline solution was used to wash the organisms from the surface of the medium and as the suspending liquid. The spore suspensions were placed in 6-oz Blake bottles containing several glass beads to aid in breaking up clumps of spores and were heated 20 min at 80°C to destroy vegetative cells. Blake bottles containing sterile physiological saline were used for diluting concentrated spore suspensions. Each spore dilution was shaken vigorously for 2 min to produce a uniform distribution of spores in the suspending liquid before making the next dilution. The spores were counted by using the most probable numbers method with 10-fold dilutions and 5 or 10 tubes per dilution. Spore suspensions were stored at 4.4°C.

Procedure for determining incidence of spores. Cans or glass jars containing meat samples were opened using aseptic precautions. Sixty-gram portions were carefully removed, weighed, and placed in sterile Waring blender containers having a capacity of 1 L. Two hundred and forty ml of sterile physiological saline solution were added to the meat, and the mixture was blended for 10 min. Then 3 series, each consisting of 5 test tubes (18×150 mm) having 10 ml of Linden thioglycollate starch medium, were respectively inoculated with 5, 0.5, and 0.05 ml of the meat-saline mixture. The first series of 5 tubes contained 1 g of meat per tube, and the second and third series respectively held 0.1 and 0.01 g of meat. In the case of very high or very low counts, it was necessary to vary the size of the inoculum. Inoculated tubes were heated 20 min at 80°C to kill the vegetative cells and were then sealed with sterile paraffin layered over the surface of the medium. The tubes were incubated 10 days at 37°C. Tubes showing visual evidence of growth and putrefactive odor were counted in each of the dilutions, and the combination of positive tubes was noted. Growth without putrefactive odor was not considered sufficient evidence of the presence of potential spoilage-producing spores.

The most probable number of spores was determined by referring the combination of positive tubes to MPN tables devised by Halvorson and Ziegler (1933, pp. 30–32). The most probable number of spores was of value, but of greater use was knowledge of the range within which the actual spore population had been established. It was possible to determine the 95% confidence limits of the spore population by using factors supplied in a table for this purpose by Cochran (1950). Using 5 tubes per dilution and 10-fold dilutions, the factor for determining the 95% confidence limits was reported to be 3.30. The upper confidence limit of the spore population was determined by multiplying the most probable number of spores by 3.3. The lower confidence limit of the spore population was found by dividing the most probable number of spores by 3.3.

All tubes were subcultured by removing three loopfuls of the old culture and inoculating them into fresh media. The tubes were heated to 80°C to destroy vegetative cells, sealed with sterile paraffin, and incubated 4 days at 37°C. Positive tubes were again noted in each series of dilutions, and the positive tube combination was referred to MPN tables to determine the most probable number of spores present in the tubes.

RESULTS AND DISCUSSION

Incidence of putrefactive anaerobic spores in meat. *Fresh pork trimmings.* Fresh pork trimmings were obtained in each of the four plants; those obtained from plant D were collected so that nearly every hour during the day's production was represented. The incidence of putrefactive anaerobic spores in fresh pork trimmings was determined by using the general method described for this purpose. Results are summarized in Table 1.

Usually, less than 0.18 putrefactive anaerobic spores per gram were recovered from fresh pork trimmings. The maximum count

Table 1. Comparison of frequencies with which various spore counts occurred in fresh pork trimmings from different plants.

Range of MPN spore count/g. meat	No. of samples containing spore count				
	Plant A	Plant B	Plant C	Plant D	Total for all plants
Below 0.18	2	1	2	18	23
0.18– 1	2	2	4	12	20
1– 2	4		3	1	8
2– 3	3	1	1		5
3– 4			2		2
7– 8		1			1
18–23			1	1	2
51				1	1
Total samples	11	5	13	33	62

of spores of any sample of pork trimmings was 51 spores per gram. The conclusion must be reached that: 1) the spore count of fresh pork trimmings is low, and 2) there is no practical difference in the spore counts of meat obtained from the different plants. The counts were so low that no differences could be ascribed to geographical, yearly, or hourly differences in the collection of samples.

Cured pork trimmings. Cured pork trimmings were secured from two packing plants in different parts of Iowa. Putrefactive anaerobic spore counts were determined by the general method. Results are summarized in Table 2. The mode of putrefactive anaerobic spores in cured pork trimmings was less than 1 per gram. The maximum spore count was 43 putrefactive anaerobic spores per gram. The spore counts, in general, were similar to those obtained from fresh pork trimmings even though the cured pork trimmings had undergone more processing than the fresh samples.

Table 2. Comparison of frequencies with which various spore counts occurred in cured pork trimmings from two plants.

Range of MPN spore count/g meat	No. of samples containing spore count		
	Plant A	Plant B	Both plants
Below 0.18	1	0	1
0.18–1	5	10	15
1–2	2	1	3
2–3	2		2
17	1		1
43	1		1
Total samples	12	11	23

Six-pound canned pork luncheon meat. Six-pound cans of pork luncheon meat were secured from two commercial packing plants. These cans had received a heat process considered insufficient to destroy spores. Therefore, it would be expected that the spore count in this product would approach that of cured pork trimmings. Results of this study are shown in Table 3. As with cured trimmings, most of these samples contained 0.18–1.0. Of samples of six-pound canned pork luncheon meat tested, 94% contained less than two spores per gram. It may be seen (Table 3) that spores count levels were similar for the two plants.

Table 3. Comparison of frequencies with which various spore counts occurred in canned pork luncheon meat (6 lb) from two plants.

Range of MPN spore count/g meat	No. of samples containing spore count		
	Plant A	Plant B	Both plants
Below 0.18	4	0	4
0.18–1	5	4	9
1–2	2	1	3
3–4	1		1
Total samples	12	5	17

Twelve-ounce canned pork luncheon meat. Commercially sterile 12-ounce cans of pork luncheon meat were secured from two packing plants. The spore count of each of 14 samples tested was less than 0.18 spore per gram. Since this product keeps at room temperature, a low spore count was expected.

Fresh beef trimmings. A limited study was carried out on the incidence of putrefactive anaerobic spores in fresh beef trimmings. Two lots of meat were secured from a retail outlet handling meat from plant E. The spore count obtained on two lots of meat are summarized in Table 4. On the basis of these data, it appears that the incidence of putrefactive spores in fresh beef is low but represents approximately a hundredfold increase over the level reported by Ayres and Adams (1953). While the latter workers reported an average load of 0.7–6.0 spores per 100 g of meat, the mean spore count for the fresh beef trimming samples run in the present study was 650 spores per 100 g of meat. Conclusions as to the incidence of putrefactive anaerobic spores in beef would not be warranted without additional tests.

Table 4. Comparison of spore counts obtained on two lots of fresh beef trimmings.

Sample no.	MPN spores/g meat	
	Lot 1	Lot 2
1	0.80	0.70
2	5.5	46
3	0.23	5.5
4	0.39	1.1
5	2.3	2.5
Total	9.22	55.80
Mean	1.8	11

Effect of preliminary enrichment of vegetative cells on spore count. The incidence of putrefactive anaerobic sporeformers in three types of meat was determined with and without preliminary heat treatment. All tubes were heated at the time of subculture so that counts reported represent the most probable number of spores as determined by combinations of positive tubes after subculture. Results are summarized in Table 5.

Table 5. Spore counts obtained with and without preliminary destruction of vegetative cells.

	Spore count/g meat			
	No preliminary heat treatment		Preliminary heat treatment	
Type of meat	MPN	95% conf. interval	MPN	95% conf. interval
Fresh pork trimmings	1.1	0.33–3.6	1.1	0.33–3.6
Cured pork trimmings	0.2	0.06–0.66	0.78	0.24–2.6
Pork luncheon meat (6 lb)	0.2	0.06–0.66	0.18	.....

The above data indicated that there was no significant difference between spore counts obtained either with or without preliminary destruction of vegetative cells. This was particularly interesting since all tubes containing fresh and cured pork trimmings were positive before subculture. The count of anaerobic vegetative cells was at least 100 cells per gram of meat. Even tubes containing processed meat (6-lb perishable pack) showed a vegetative content of 30 cells per gram of meat before subculture. Of this number, none of those capable of surviving subculture were able to form spores and contribute to the count. Therefore, most of the vegetative cells present were facultative anaerobic rods.

To follow the process under controlled conditions, a known load of spores of *Clostridium* sp. (National Canner's Association's putrefactive anaerobe PA3679) was mixed with fresh pork trimmings in a Waring blender. The mixture was used to inoculate a series of MPN tubes that were incubated without preliminary heat treatment. In order that the inoculated spores be recovered in the highest dilutions, it was necessary that they germinate, multiply, and again sporulate in the presence of a variety of anaerobic vegetative cells which outnumbered the spores added. Results are presented in Table 6.

Table 6. Recovery of a known load of *Clostridium* sp. PA 3679 spores from fresh unheated pork trimmings.

	MPN spores/g meat	95% conf. interval
Spore count of meat alone	1.1	0.33– 3.6
Spores added to meat	1.0	0.43– 2.3
Spore count [a] of inoculated meat	3.2	0.97–11.0

[a] Spore count determined after subculture; subculture tubes heated 20 min at 80°C to destroy vegetative cells.

Table 6 reveals that inoculated spores apparently were recovered and that preliminary enrichment of anaerobic vegetative cells in meat failed to increase the count of putrefactive anaerobic spores. It is possible that the putrefactive anaerobic vegetative cells may have been selectively destroyed by oxygen during the mixing process, but it was shown earlier that a high count of anaerobic vegetative cells did come through the mixing procedure. It is of interest that few of these cells formed spores. This, again, is an indication that the vegetative cells present were mainly facultative anaerobes.

The above experiment also indicated that vegetative facultative anaerobic cells did not interfere with germination, multiplication, sporulation, and recovery of spores of the obligate anaerobe PA 3679.

Recovery of inoculated spore loads. A method for evaluating the counting procedure was to add known numbers of putrefactive anaerobic spores to the meat and to determine their percentage recovery. This procedure was satisfactory, assuming that the meat did not inhibit development of the organisms. If all inoculated viable spores were not recovered, either the counting method was in error or the meat inhibited development of the spores.

Dilutions of meat and known numbers of spores were mixed in two ways for recovery studies: 1) directly in tubes of media, and 2) in a Waring blender. The first method required separate inoculation of dilutions of

meat and of spores into tubes of media; the second method necessitated only a single inoculation into tubes since the spores and the meat were already combined, and it also brought the meat and spores into more intimate contact than did the first method. Results were similar with either procedure, and in later work the Waring blender was used exclusively. The general method was to determine spore counts on: 1) the spore suspension, 2) the meat, and 3) the combination of meat and spores. If the count of spores in the combination was equal to the sum of the spores naturally present in the meat and the spores added to the meat, recovery was considered complete. Typical recoveries are cited in Table 7.

On the basis of numbers of spores added to and numbers of spores recovered from fresh and cured pork trimmings, no inhibition was evidenced by these two types of meat. Therefore, counts of putrefactive anaerobic spores in fresh and cured pork trimmings, using the general method, may be considered to be valid estimates of the spore population.

Recoveries of inoculated loads of PA 3679 spores were not as complete from canned pork luncheon meat as from fresh and cured pork. The range of recoveries was 20–100% when the 12-oz commercially sterile canned pork luncheon meat was tested. Recoveries from 6-lb perishable canned pork luncheon meat were approximately 40–45%. In all cases, the inhibition was insignificant if the overlapping of the 95% confidence intervals was used as a basis. Although the validity of spore counts on pork luncheon meat may be questioned on the basis of the recovery studies cited in Table 7, it is doubtful whether the inhibition has practical significance. The lowest recovery obtained was 20% of the inoculated spore load. The highest spore count obtained on any can of pork luncheon meat tested was 3.3 spores per gram. Assuming that this count represents only 20% of the viable spores present, the total spore count would be 17 spores per gram of meat. Using the general method for determination of incidence of putrefactive anaerobic spores in meat, the 95% confidence intervals for a population of 3.3 and 17 spores are 0.10–11 and 5.2–56 spores, respectively. In view of the overlapping of the intervals, differences between such counts are not significant.

Various workers, including Hoffman *et al.* (1939), Kodicek and Worden (1945), Humfeld (1947), and Foster and Wynne (1948) have reported that the unsaturated fatty acids inhibit microorganisms. Also, Foster and Wynne (1948) and, later, Hardwick *et al.* (1951) indicated that, during extraction, the saturated C_{10}–C_{14} fatty acids and the unsaturated oleic, linolenic, and linoleic acids that are released may exert toxicity. To determine if the fatty components in pork were responsible, at least in part, for the inhibition of spore germination and outgrowth, pork luncheon meat from the commercially sterile 12-oz can was extracted with ether in a Soxhlet extractor. The ether-soluble fraction—after removal of ether—was added to a known number of PA 3679 spores in a concentration equivalent to that present in meat. As shown in the last item in Table 7, only 5% of the inoculated spores were recovered. There was a significant inhibition of spore germination and outgrowth in these trials.

Since the concentration of ether-soluble materials added to the spores was equivalent to that present in meat samples added to like numbers of spores, it would be of value to know why the inhibition was greater after extraction than before. One explanation (Roth and Halvorson, 1952) is that the fats become more oxidized and thus more toxic as they are being extracted. Another is that, within the meat, the inhibitory compounds are in combination with other materials and are less able to exert their full effect.

General discussion. Since the usual number of putrefactive anaerobic spores in fresh pork trimmings was less than 0.18 and less than 1 per gram in both cured pork trimmings and canned pork luncheon meat, the general level of contamination appears to be very low. These results extend the work of Burke *et al.* (1950), who reported an average of less than one spore per gram of pork, and of Harriman *et al.* (1948), who found an average of 2–4 spores per gram of pork.

Gross *et al.* (1946) investigated the effect

Table 7. Recovery of known numbers of PA 3679 spores from pork trimmings and from an ether-soluble extract of canned pork.

	Spores/g meat						
Type meat	Meat MPN	95% conf. interval	Spores added MPN	95% conf. interval	Combination meat+spores MPN	95% conf. interval	Recovery basis of MPN (%)
Fresh	<0.18		4.7	2.0– 11.	6.6	2.8 – 15.	140
Fresh	0.20	0.086–0.46	12.	5.2– 28.	17.	7.4 – 39.	139
Fresh	0.20	0.086–0.46	35.	15. – 81.	54.	23. –130.	150
Cured	0.20	0.086–0.46	4.7	2.0– 11.	6.6	2.8 – 15.	135
Cured	0.40	0.17 –0.93	120.	52. – 280.	120.	52. –280.	100
Canned (6 lb)	0.20	0.061–0.66	5.0	1.5– 17.	2.3	0.70– 7.6	44
Canned (6 lb)	0.20	0.061–0.66	54.	16. – 180.	22.	6.7 – 73.	41
Canned (12 oz)	<0.18		8.6	3.7– 20.	2.8	1.2 – 6.5	33
Canned (12 oz)	<0.18		3.5	1.5– 8.1	3.5	1.5 – 8.1	100
Canned (12 oz)	<0.18		3.5	1.5– 8.1	1.7	0.74– 3.9	49
Canned (12 oz)	<0.18		3.5	1.5– 8.1	1.5	0.65– 3.5	43
Canned (12 oz)	<0.18		3.5	1.5– 8.1	2.4	1.0 – 5.6	69
Canned (12 oz)	<0.18		6.0	2.6– 14.	1.2	0.52– 2.8	20
Canned (12 oz)	<0.18		30.	13. – 70.	6.0	2.6 – 14.	20
Canned (12 oz)	<0.18		600.	260. –1400.	170.	73. –390.	28
Canned (12 oz)	<0.18		15.	6.5– 35.	12.	5.2 – 28.	80
Ether-soluble fraction (12 oz)	<0.18		15.	6.5– 35.	0.85	0.37– 2.0	5

that the numbers of spores of PA 3679 suspended in meat have on destruction times at 121.1°C. They stated that the heat resistance of PA 3679 is well above that of any bacterium naturally occurring in or contaminating pork. Data available in the literature were summarized, and the destruction times were compared with the numbers of spores used. A curve was drawn that is described by the equation $y = Ae^{Bx}$, in in which A, B, and e are constants, y is the number of spores per gram of meat, and x is the thermal process value expressed as the number of minutes required to destroy the spores at 121.1°C, assuming instantaneous heating and cooling. The values of the constants were: $A = 0.000056$, $B = 2.02$, and $e = 2.7$ (base of the Naperian logarithms). The formula is more readily employed in the form:

$$\log \frac{y}{A} = \frac{Bx}{2.3}$$

In addition to calculating the theoretical destruction times for various concentrations of PA 3679 spores, Gross *et al.* determined experimental destruction times for several spore concentrations. Table 8 was constructed from data presented by Gross *et al.* (1946). As the table illustrates, the thermal death time decreased with lower concentrations of PA 3679 spores. At least for concentrations of ten and of 10,000 spores per gram of meat, theoretical calculations using the formula agreed very well with experimental thermal death times. Thus, it appears

Table 8. Thermal death time for various concentrations of PA 3679 spores in meat.

Spores/g meat (y)	Theoretical thermal death time (x) (min at 121.1°C)	Experimental thermal death time (min at 121.1°C)
1,000,000,000	15.1	....
10,000	9.4	9.5
10	6.0	6.0
3	5.4	....

that a TDT of the order of 6.0 min rather than 15.1 min at 121.1°C is more in keeping with conditions requisite for freeing meat of these organisms.

The present investigation indicates that incidence of putrefactive anaerobic spores in meat is low. If these findings reflect conditions prevailing throughout the meat industry, future thermal death times studies with putrefactive anaerobic spores should involve use of spore loads approximating those normally occurring in meat.

REFERENCES

Ayres, J. C., and A. T. Adams. 1953. Occurrence and nature of bacteria in canned beef. *Food Technol.* **7**, 318.

Burke, M. V., K. H. Steinkraus, and J. C. Ayres. 1950. Methods for determining the incidence of putrefactive anaerobic spores in meat products. *Food Technol.* **4**, 21.

Cochran, W. G. 1950. Estimation of bacterial densities by means of the "most probable number." *Biometrics* **6**, 105.

Foster, J. W., and E. S. Wynne. 1948. Physiological studies on spore germination, with special reference to Clostridium botulinum. IV. Inhibition of germination by unsaturated C_{18} fatty acids. *J. Bacteriol.* **55**, 495.

Gross, C. E., C. Vinton, and C. R. Stumbo. 1946. Bacteriological studies relating to thermal processing of canned meats. VI. Thermal death-time curve for spores of test putrefactive anaerobe in meat. *Food Research* **11**, 411.

Halvorson, H. O., and N. R. Ziegler. 1933. "Quantitative Bacteriology." Burgess Publishing Co., Minneapolis, Minn.

Hardwick, W. A., B. Guirard, and J. W. Foster. 1951. Antisporulation factors in complex organic media. II. Saturated fatty acids as antisporulation factors. *J. Bacteriol.* **61**, 145.

Harriman, L. A., V. J. DelGiudice, B. M. Shinn, and R. Hansen. 1948. The incidence of putrefactive anaerobes in pork. Illinois Bacteriologists, fall meeting. Peoria, Ill. Oct. 15, 1948. (abstract of paper with meeting notice).

Hoffman, C., T. R. Schweitzer, and G. Dalby. 1939. Fungistatic properties of the fatty acids and possible biochemical significance. *Food Research* **4**, 539.

Humfeld, H. 1947. Antibiotic activity of the fatty-acid-like constituents of wheat bran. *J. Bacteriol.* **54**, 513.

Kodicek, E., and A. N. Worden. 1945. The effect of unsaturated fatty acids on Lactobacillus helveticus and other gram-positive micro-organisms. *Biochem J.* **39**, 78.

Roth, N. G., and H. O. Halvorson. 1952. The effect of oxidation rancidity in unsaturated fatty acids on the germination of bacterial spores. *J. Bacteriol.* **63**, 429.

The Relative Importance of Chicken Egg Exterior Structures in Resisting Bacterial Penetration

A. LIFSHITZ, R. C. BAKER, AND H. B. NAYLOR
Cornell University, Ithaca, New York

(Manuscript received May 31, 1963)

SUMMARY

Three sets of egg shell models were devised and used to measure the mean resistance time of each of the three exterior structures of the egg to penetration by *Pseudomonas fluorescens*. It was found that the inner shell membrane is the most important barrier, the shell ranks second, and the outer membrane is the least important.

INTRODUCTION

The exterior structures of the egg (shell and outer and inner shell membranes) constitute a set of barriers that bacteria must pass to enter the egg. Elliott (1954) inoculated bacteria into the air cell of eggs and showed that 4–8 days elapsed before the bacteria were isolated from the egg contents. Walden *et al.* (1956) used *Pseudomonas fluorescens* labeled with P^{32} for penetration studies. Their results showed that the membranes resisted the bacteria for 20 hr. Then the radioactivity of the filtrate from the shell increased rapidly, indicating that the membranes had ruptured and allowed passage of bacteria. Kraft *et al.* (1958) and Garibaldi and Stokes (1958) showed that the membranes are more important than the shell in hindering the entrance of bacteria into the egg, but they treated the inner and outer shell membranes as one unit and did not differentiate between their protective abilities. No work has been done on the relative importance of all these egg barriers, especially on the relationships of the membranes. A comparative study of these structures was therefore conducted to illuminate their importance and help explain differences in the resistance to bacterial penetration of eggs from different avian species and among strains of chickens.

MATERIALS AND METHODS

Eggs of one strain of Single-Comb White Leghorns were used within 24 hr of lay. Eggs were washed with a sanitizing detergent, dried, and stored at 2°C. Before being processed, the eggs were immersed in a 1% solution of mercuric chloride for one minute and with 70% alcohol for two minutes to eliminate superficial bacteria. They were then coated with paraffin wax. Models were prepared as shown in Fig. 1.

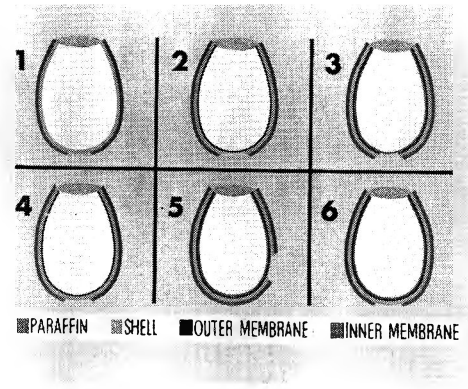


Fig. 1. Models coated with wax for studying bacterial penetration through the exterior structures (Experiment 1).

Model 1. A circular cut was made at the small end of each egg with an electric cutter (Dremel kit Model No. 2) and the contents discarded. With a sterile spatula the membranes were removed; care was taken to avoid harming the shell. An area of one sq cm of the shell at the bottom was cleared from the paraffin. *This model had the shell as the only barrier.*

Model 2. The shell in the area of the air cell was carefully peeled off with a scalpel. The egg was then cut as described in the previous model and the contents discarded. It was then washed with sterile water. With vacuum the inner membrane was sucked out. *This model had the outer membrane as the only barrier.*

Model 3. The shell and the outer membrane in the air cell region were removed with a scalpel. The egg was then cut at the small end as described

and the contents discarded. It was then washed with sterile water. *This model had the inner membrane as the only barrier.*

Model 4. The egg was cut in the small end and the contents were discarded. The inner membrane was sucked out and the inside of the egg was washed with sterile water. An area of one sq cm of the shell at the bottom was cleared from the paraffin. *This model had the shell and the outer membrane as barriers.*

Model 5. A window was cut in the egg near the air cell. The shell was cautiously removed without damaging the outer membrane. The egg was then cut, the contents discarded, and the interior washed well with sterile water. *This model had both inner and outer membranes as barriers.*

Model 6. The egg was cut, the contents were discarded, and the interior was washed well with sterile water. An area of one sq cm of the shell at the bottom was cleared from the paraffin. *This model had all three structures as barriers.*

Twenty sets of each of the six models were prepared. Each set of six models was put into a desiccator as shown in Fig. 2. Sterile proline

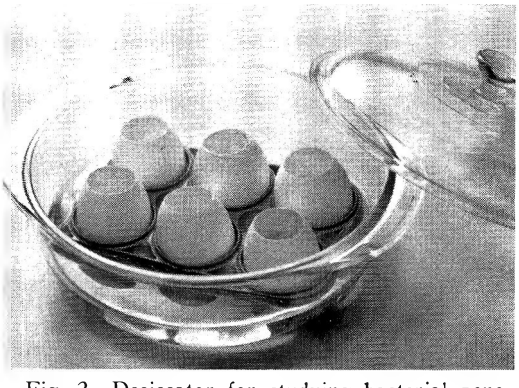


Fig. 2. Desiccator for studying bacterial penetration.

broth (3 g l-proline, 0.5 g $MgSO_4$, and 0.5 g K_2HPO_4 per liter) was added to the models in their stands in the desiccators. The same broth was added to the desiccators outside the models until the level of the broth was about 2 cm below their top openings. All of this was done aseptically. A sterility test was performed afterward by incubating the desiccators with the models for 48 hr at 30°C. Then samples were taken from the desiccators, examined under the microscope, and streaked on agar. Whenever a desiccator showed signs of contamination, it was rejected. The sterile desiccators were inoculated with a high concentration of bacteria to give a final titer of about 1×10^9 *Pseudomonas fluorescens* cells per milliliter. Samples from the broth inside the models were taken at zero time and at every hour for 25 hr and streaked on agar. Plates were incubated at 30°C and observed after 24–48 hr.

In Experiment No. 2, another type of model was used as shown in Fig. 3. In these models, all

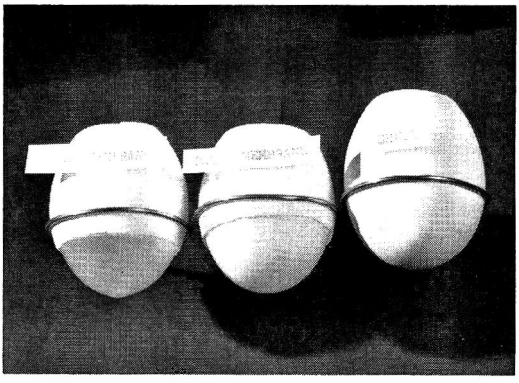


Fig. 3. Large-area models used in Experiment 2.

of the lower part of the egg, the part immersed in the broth, was exposed, giving a larger area of contact between the bacteria and the barrier under study. Models were prepared as follows:

Model 1. This model was identical to and was prepared in exactly the same way as model 1 for Experiment 1, except for the paraffin coating. *The resistance to bacteria was offered by the shell only.*

Model 2. After the alcohol evaporated, the egg was cut, the contents discarded, and the inner membrane sucked out completely. The egg was then put into a solution of heat-sterilized disodium ethylenediaminetetraacetate (EDTA) (pH adjusted with concentrated sodium hydroxide to 7.5) in a sterile desiccator. After 1 hr the model was taken out and the shell easily removed. The model was then washed thoroughly with sterile water and immersed in a closed sterile container of water until used. *This model had only the outer membrane to resist bacteria.*

Model 3. This model presented the most problems in sterility. About every fourth model had to be discarded because it was not sterile. Preparation was as follows: After the egg was cut and the contents discarded, the model was washed with sterile water to eliminate any adhering albumen. The inner membrane was then sucked halfway up. With the cutter, the bottom of the egg from which the inner membrane had been separated was now cut. Sterile water was added afterward to the interior of the egg, causing the inner membrane to return to its original and natural place. It was then stored in a sterile desiccator until used. *This model had the inner membrane as the only barrier to bacteria.*

Model 4. This model was identical to and was prepared in exactly the same way as model 4 for Experiment 1, but for the paraffin coating. *The resistance to bacterial penetration was offered by the shell and the outer membrane.*

Model 5. After allowing the alcohol to evaporate, the egg was cut, the contents discarded, and the shell washed thoroughly with sterile water. The shell was then put into a sterile solution of EDTA in a sterile desiccator. It was taken out after 1 hr, and the lower part of the shell was easily removed. The model was then washed with sterile water and kept in a sterile container until used. *This model had the outer and inner membranes as barriers to bacterial penetration.*

Model 6. This model was identical to and prepared in the same way as model 6 for Experiment 1, except for the paraffin coating. *The resistance to bacterial penetration was offered by the shell and inner and outer shell membranes.*

Six eggs, one of each model, were placed in each of five desiccators. Broth was poured into the egg models and into the desiccator outside the eggs. Bacteria were added to give a final titer of about 1×10^6 *Pseudomonas fluorescens* per milliliter to the outside broth. Samples were taken each hour for 15 hr and then at 22 and 30 hr.

Experiment 3 was designed to simulate "in vivo" conditions, i.e., using whole eggs rather than

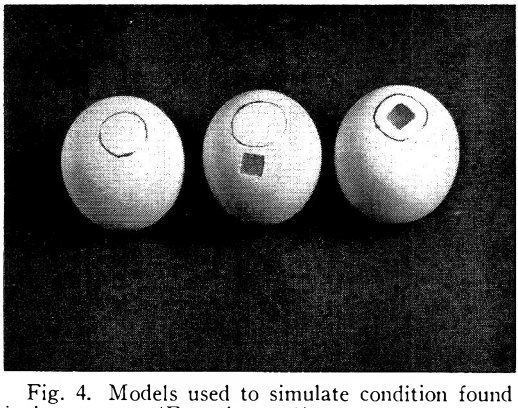


Fig. 4. Models used to simulate condition found in intact eggs (Experiment 3).

dissected models. In this experiment, penetration from the inside out was studied in the intact egg. Only three models could be used here, because it is impossible to remove the membrane and keep the egg whole. The models shown in Illustration 3 were prepared in the following way:

Model 1. After evaporation of the alcohol, the shell and the outer membrane in the air cell area were removed with the aid of a sterile scalpel. Afterward, the egg was swabbed with mercuric chloride and alcohol and was then put into a sterile desiccator until inoculated. *In this model, the resisting barrier was only the inner membrane.*

Model 2. After evaporation of the alcohol, a window was cut outside of the air cell, and the shell was removed. The shell parts were swabbed with mercuric chloride and alcohol. Care was taken not to touch the exposed membranes. The egg was stored in a sterile desiccator until inoculated. *In this model, the resistance was composed of both inner and outer membranes.*

Model 3. This was the untreated whole egg. *In this model, the resistance was composed of all the three outer structures—shell, inner membrane, and outer membrane.*

The models were taken from a sterile desiccator, where they had been stored. With a sterile needle, a 1-mm hole was made in the shell at the small end of the egg (the outer membrane was not harmed). Two-tenths of a milliliter of a 24-hour culture of *Pseudomonas fluorescens* were injected into the egg with a sterile tuberculin syringe. The hole was closed with paraffin, and each egg was put into a wide-mouth Erlenmeyer flask, which contained sterile albumen prepared and tested as described by Garibaldi and Stokes (1958).

Thirty eggs, ten of each model, were prepared. The 30 flasks were incubated at 30°C, and plates were streaked every 6 hr for 120 hr. Albumen, rather than broth, had to be used as an outer medium because of the high osmotic pressure existing in this system. When broth was added outside of models 1 and 2, it caused them to burst.

RESULTS AND DISCUSSION

Results of the first experiment are shown in Table 1. From these results the mean resistance time for each treatment was calculated, as shown in Table 2.

Analysis of variance for the first three treatments is given in Table 3. The results of this experiment show that the inner membrane is the most important single barrier to bacterial penetration into the egg. Its contribution to the entire resistance of the egg is significantly higher and different from that of the other exterior structures. Kraft *et al.* (1958) showed that the membranes are the important barriers, but did not differentiate between the inner and outer membranes. Elliot (1952) inoculated *Pseudomonas fluorescens* into the air cell and showed that it took about four days for the bacteria to be detected inside of the egg, but he did not evaluate the resistance of the other barriers. Garibaldi and Stokes (1958)

Table 1. Bacterial penetration into six egg shell models (number of samples of each model penetrated at various times).

Time of measurement (hr)	Models [a]					
	A	B	C	A+B	B+C	A+B+C
1	1	1				
2	3	3				
3	4	5				
4	6	9		1		
5	7	14		1		
6	8	16		3		
7	9	17		3		
8	9	19	1	5		
9	9	20	2	5	2	1
10	13		3	7	5	2
11	14		5	7	5	2
12	16		9	9	5	2
13	17		12	9	8	2
14	19		14	13	12	4
15	20		14	13	12	4
16			16	15	14	8
17			16	15	14	8
18			18	16	16	8
19			19	18	17	8
20			20	20	17	12
21					18	14
22					18	15
23					19	15
24					20	16
25						18
30						20

[a] A, shell; B, outer shell membrane; C, inner membrane.

Table 2. Mean resistance time of the outer structures to bacterial penetration.

Models [a]																		
A			B			C			A+B			B+C			A+B+C			
n	t	nt	n	t	nt	n	t	nt	n	t	nt	n	t	nt	n	t	nt	
1	15	15	1	9	9	1	20	20	2	20	40	1	24	24	2	30	60	
2	14	28	2	8	16	1	19	19	2	19	38	1	23	23	2	25	50	
1	13	13	1	7	7	2	18	36	1	18	18	1	21	21	1	24	24	
2	12	24	2	6	12	2	16	32	2	16	32	1	19	19	1	22	22	
1	11	11	5	5	25	2	14	28	4	14	56	2	18	36	2	21	42	
4	10	40	4	4	16	3	13	39	2	12	24	2	16	32	4	20	80	
1	7	7	2	3	6	4	12	48	2	10	20	4	14	56	4	16	64	
1	6	6	2	2	4	2	11	22	2	8	16	3	13	39	2	14	28	
1	5	5	1	1	1	1	10	10	2	7	14	3	10	30	1	10	10	
2	4	8	20		96	1	9	9	1	4	4	1	9	9	1	9	9	
1	3	3				1	8	8	20		262	1	8	8	20		389	
2	2	4				20		271				20		297				
1	1	1																
20		165																
Mean:																		
8.3			4.8			13.6			13.1			14.9			19.5			

The number of samples (n) that resisted the bacteria for a certain number of hours (t) was multiplied by that number of hours. The figures were added up and divided by the twenty samples.

[a] A, shell; B, outer shell membrane; C, inner membrane.

Table 3. Analysis of variance for Experiment 1.

Source	d.f.	S.S.	M.S.	F
Treatment	2	777	388.5	32.1**
Error	57	690	12.1	
Total	59	1467		

LSD (Tukey) = $W = q\alpha\ (p \cdot n_2)S\bar{\bar{x}} = 4.28 \times 0.78 = 3.34$.

The three means are significantly different at the 1% level.

showed that the shell with the outer membrane on was much more effective in resisting bacterial penetration than the shell without the membranes, but his results were obtained using filtration techniques with a vacuum of 460 mm Hg.

The main concern in this experiment was the three individual exterior structures. However, results show that the inner membrane alone (model 3) is at least as good a barrier as both the shell and the outer membrane together (model 4).

Results of Experiment 2 are shown in Table 4. Because of lack of continuity of sampling (no samples were taken between 15 and 22 hours), it was impossible to calculate the mean resistance time for the different models of this set and to evaluate the results. It can be seen, however, that they agree with the results of the previous experiment at least qualitatively.

Results of the third experiment are shown in Table 5, the mean resistance time in Table 6, and the analysis of variance in Table 7. Results of this experiment indicate that conclusions drawn from the previous experiments in which somewhat unnatural models were used also apply to

Table 5. Bacterial penetration out of three "in vivo" egg models (number of samples penetrated at various times).

Time of measurements (hr)	Sampling no.	Models [a] B	B+C	A+B+C
6	1			
12	2			
18	3			
24	4	1	1	
30	5	2	1	
36	6	4	2	1
42	7	5	3	1
48	8	5	5	1
54	9	7	5	3
60	10	8	6	4
66	11	9	6	5
72	12	9	7	5
78	13	10	7	7
84	14		8	7
90	15		9	8
96	16		10	8
102	17			9
108	18			10

[a] A, shell; B, outer shell membrane; C, inner shell membrane.

Table 4. Bacterial penetration into six egg shell models with large area exposed (number of samples of each model penetrated at various times).

Time of measurement (hr)	Models [a] A	B	C	A+B	B+C	A+B+C
1						
2						
3	1					
4	1	2				
5	1	2				
6	2	3				
7	2	3		1		
8	2	5		2		
9	3			2	1	
10	4		1	2	1	
11	4		1	3	1	
12			1	3	1	1
13			1	3	2	1
14			2	3	2	2
15			2	4	2	2
22			5	5	4	4
30					5	5

[a] A, shell; B, outer shell membrane; C, inner shell membrane.

Table 6. Mean resistance time of three "in vivo" models.

C			B+C			A+B+C		
n	t	nt	n	t	nt	n	t	nt
1	13	13	1	15	15	1	18	18
1	12	12	1	14	14	1	17	17
1	10	10	1	13	13	1	15	15
2	9	18	1	11	11	2	13	26
1	7	7	1	9	9	1	11	11
2	6	12	2	7	14	1	10	10
1	5	5	2	6	12	2	9	18
1	4	4	1	4	4	1	6	6
10		81	10		92	10		121
Mean resistance time:								
8.1			9.2			12.1		

Table 7. Analysis of variance, Experiment 4.

Source	d.f.	S.S.	M.S.	F
Treatment	2	85	42.5	3.33
Error	27	344	12.74	
Total	29	429		

Not significant (at 5% level).

whole eggs. The inner membrane is the most important single barrier to bacterial penetration of the egg.

There was no significant difference between the mean resistance time of the inner membrane alone (C) and the other two models which contained the inner membrane: the outer and inner (B+C), and all three structures (A+B+C). This indicates that the relative contributions of the outer membrane and the shell are relatively small.

REFERENCES

Elliott, R. P. 1958. Spoilage of shell eggs by Pseudomonas. *Appl. Microbiol.* **2**, 158.

Garibaldi, J. A., and J. L. Stokes. 1958. Protective role of shell membranes in bacterial spoilage of eggs. *Food Research* **23**, 283.

Kraft, A. A., L. E. Elliott, and A. W. Brant. 1958. The shell membranes as a barrier to bacterial penetration of eggs. *Poultry Sci.* **37**, 238.

Walden, C. C., I. V. F. Allen, and P. C. Trussell. 1956. The role of egg shell and shell membranes in restraining the entry of microorganisms. *Poultry Sci.* **35**, 1190.

Biochemical and Serological Relationships of Putrefactive Anaerobic Sporeforming Rods Isolated from Pork [a]

K. H. STEINKRAUS [b] AND J. C. AYRES
Departments of Bacteriology and Dairy and Food Industry, Iowa State University, Ames, Iowa

(Manuscript received April 11, 1963)

SUMMARY

Under anaerobic culture conditions, 120 cultures of sporeforming rods were isolated from fresh and cured pork trimmings and from pork luncheon meat; 22 of these proved to be *obligate* anaerobic putrefactive organisms. Studied by the Reed and Orr method of rapid identification, the following species were identified: *Clostridium tetanomorphum*, *Cl. novyi*, *Cl. carnis*, *Cl. paraputrificum*, *Cl. tetani*, *Cl. histolyticum*, and *Cl. sporogenes*. One culture was similar to *Clostridium* sp. National Canners Association putrefactive anaerobe 3679 (PA 3679). Serological relationships were determined by using antisera for *Cl. sporogenes* and PA 3679. No cross agglutination was obtained between *Cl. sporogenes* and PA 3679. An organism identified biochemically as *Cl. carnis* agglutinated in dilutions of 1:5120 of PA 3679 antiserum, indicating a very close serological relationship. Several organisms revealed antigens in common with *Cl. sporogenes*. The organism with biochemical reactions similar to PA 3679 showed no serological relationship to the known species. Serological relationships correlated with heat resistance while biochemical reactions did not.

INTRODUCTION

As part of a study on the incidence of putrefactive anaerobic spores in pork trimmings (Burke et al., 1950; Steinkraus and Ayres, 1963), 120 cultures of typical sporeforming spoilage organisms were collected. These cultures were purified and investigated biochemically and serologically in an effort to characterize typical species of obligate anaerobes occurring in pork.

EXPERIMENTAL METHODS

Purification and determination of oxygen requirements. Serial dilutions were made of each of the isolated cultures in tubes of freshly exhausted anaerobic agar (Baltimore Biological Laboratories, Inc.) containing 0.1% soluble starch to which 1:200,000 crystal violet was added to inhibit facultative anaerobes (Soc. of Am. Bacteriologists, 1957, p. 133). These dilutions were poured in Krumwiede-Pratt plates (Tanner, 1933); as soon as the medium had solidified, the plates were sealed by applying a sterile mixture of mineral oil and melted paraffin in the space between the edges of the inner and outer dishes. At the same time, similar dilutions were made in melted agar containing 2% trypticase and 0.5% NaCl but no reducing agents. These dilutions were poured in aerobic Petri dishes. Both series of plates were incubated 5 days at 37°C. Subcultures of the highest dilutions were made whenever necessary to ensure the purity of the cultures.

Organisms that were indiscriminate in their oxygen tension requirements were considered to be facultative anaerobes and were excluded from the study. A stained smear was made of the obligate anaerobes to confirm that the organisms were Gram-positive and in the sporeforming stage. Then each culture was transferred to Linden's thioglycollate medium (Difco) to which 0.1% soluble starch was added, and the tube was heated 20 min at 80°C prior to incubation so that vegetative cells would be destroyed.

Biochemical identification. When visible growth was observed on incubation at 37°C, a 0.1-ml inoculum was introduced in the bottom of tubes

---

[a] Journal Paper No. J-4578 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project No. 1264; Center for Agricultural and Economic Development Cooperating.

This paper reports research undertaken in cooperation with the Armed Forces Food and Container Institute, U. S. Army Quartermaster Research and Engineering Center, and has been assigned number 2275 in the series of papers approved for publication. The views or conclusions contained in this report are those of the author(s). They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

[b] Present address: Dept. of Food Science and Technology, Cornell University, Geneva, New York.

of each of the media employed in the Reed and Orr (1941) method for identification of anaerobes. Each test was performed at least three times; when reactions were irregular, several additional checks were made. The biochemical patterns displayed by the organisms were related to those characteristics of various species of the genus *Clostridium* as described by Reed and Orr (1941) or to those described by Gross et al. (1946a). Bergey's "Manual of Determinative Bacteriology" (Breed *et al.*, 1957) was consulted when descriptions in the literature were inadequate.

Serological relationships. Serological relationships among the organisms were studied by procedures given in the "Manual of Microbiological Methods" (Soc. of Am. Bacteriologists, 1957). The serological patterns of the various anaerobes isolated were related to the antigenic structure of two organisms that have frequently been implicated in the spoilage of canned meat, i.e., *Clostridium sporogenes* and *Clostridium* species, National Canner's Association putrefactive anaerobe strain no. 3679 (PA 3679). Antisera for these two species and two related strains were prepared by rabbit inoculation.

Antigens were prepared by growing organisms on non-antigenic glucose-beef extract media. The inoculated tubes were incubated 48 hr at 37°C in an atmosphere of specially purified "Seaford" nitrogen in Mason jars by a method described by Ogilvy et al. (1950). Rabbits were inoculated intravenously with an initial injection of 0.5 ml of the broth culture. The injections were increased by 0.5 ml at 6-day intervals until the final inoculum consisted of 3 ml of broth culture introduced peritoneally. After the last injection, six days were allowed for the development of antibodies. Using aseptic precautions, 25 ml of blood were removed from the heart of each rabbit, and the blood was allowed to clot. The sera were transferred to sterile containers and stored at 4.4°C until used in agglutination tests.

Serological relationships among these organisms and other putrefactive anaerobic sporeformers were determined by a macroscopic agglutination procedure in which a constant amount of bacterial suspension (antigen) was mixed with a series of increasing dilutions of antiserum. Agar slants of cultures that were to serve as antigens were incubated at 37°C; after growth was evident, a smooth suspension of the organisms was prepared in physiological saline.

The following serum dilutions were used: 1:20, 1:40, 1:80, 1:60, 1:320, 1:640, 1:1280, 1:2560, and 1:5120. After addition of the antigen, the tubes (13 × 125 mm) were shaken and incubated for 4 hr at 52°C. The tubes were stored overnight in a refrigerator and read the following morning. By this procedure, both H (flagellar) and O (somatic) antigens were involved in agglutination reactions.

In reading the agglutination tests, it was essential to determine that the control tube containing no antiserum still showed uniform cloudiness without sedimentation. If this proved to be the case, then the other tubes were examined for amount of sediment, granulation, and clumping. Based upon the total reaction, the tubes were rated +++, ++, +, or 0.

In selecting rabbits for immunization, care was taken that their sera did not contain natural antibodies toward the organisms to be used for antigens. Following injection of cells of the proper organism, antibodies were produced in the rabbit's serum which, when added to a suspension of the organism, caused it to agglutinate. Rough relationships of various organisms to the injected strain were determined by noting the titers, i.e., the highest dilutions of antiserum causing the organisms to agglutinate. When organisms showed a close relationship, absorbed sera were prepared and used to determine common antigenic components.

RESULTS AND DISCUSSION

Screening tests of 120 cultures of putrefactive sporeforming organisms growing anaerobically showed only 22 to be typical obligate anaerobic strains. The 22 strains of anaerobes appeared to belong to 8 distinct species (Table 1). Strains 7 and 73 differed in their response to sucrose, salicin, and gelatin, whereas strains 74 and 75 were similar biochemically except for ability to ferment salicin.

Reactions of organisms in PA 3679 antiserum. According to Gross *et al.* (1946b), PA 3679 has shown the highest heat resistance of any mesophilic putrefactive bacterium occurring naturally in or contaminating canned meat. Since PA 3679 was originally isolated from meat products and since organisms similar to this strain are occasionally found as the cause of canned meat spoilage, the serological relations between this organism and other species isolated from meat were studied. At the same time, known cultures of several anaerobic species were tested for agglutination in PA 3679 antiserum. Results are summarized in Table 2.

The only cells showing a strong agglutination reaction in PA 3679 antiserum were those of organism No. 17, isolated from meat. The latter was tentatively identified,

Table 1. Biochemical relationships of organisms isolated from pork trimmings with known species of *Clostridium*.

Organism no.	Species as characterized by Reed and Orr (1941) or Gross *et al.* (1946a)	Differences in biochemical reactions from those reported
4	*Cl.* species, PA 3679	None
7	*Cl. tetanomorphum*	Sucrose fermentation variable
16	*Cl. novyi*	None
17	*Cl. carnis*	None
26	*Cl. paraputrificum*	Hydrogen sulfide positive; gelatin liquefied
27	*Cl. tetani*	None
31	*Cl. histolyticum*	Dextrose and maltose variable
73	*Cl. tetanomorphum*	Salicin fermentation variable; gelatin liquefied
74	*Cl. sporogenes*	Salicin fermentation variable
75	*Cl. sporogenes*	None

on the basis of its biochemical reactions, as *Clostridium carnis*. Organism No. 4, which had biochemical reactions identical with PA 3679, failed to agglutinate in any dilution of PA 3679 antiserum used. In addition, absorption of PA 3679 antiserum with cells of organism No. 4 failed to reduce the titer. Therefore, no serological relation was indicated.

It is not known why spores exhibit different abilities to survive heat processes. Undoubtedly heat resistance is intimately related to the chemical structure of the spore. It is possible that serological relationships

Table 2. Agglutination reactions of various anaerobic species in PA 3679 antiserum.

Organisms	Serum dilution									
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	Control
PA 3679	+++	+++	+++	+++	+++	+++	+++	++	++	0
Clostridium sporogenes (Spray)	0	0	0	0	0	0	0	0	0	0
No. 17	+++	+++	+++	+++	+++	+++	+++	+++	+++	0
No. 4	0	0	0	0	0	0	0	0	0	0
No. 7	+	0	+	0	0	0	0	0	0	0
No. 26	0	0	0	0	0	0	0	0	0	0
No. 27	0	0	0	0	0	0	0	0	0	0
No. 31	0	0	0	0	0	0	0	0	0	0
No. 73	0	0	0	0	0	0	0	0	0	0
No. 74	0	0	0	0	0	0	0	0	0	0
No. 75	0	0	0	0	0	0	0	0	0	0
Cl. novyi	0	0	0	0	0	0	0	0	0	0
Cl. perfringens	0	0	0	0	0	0	0	0	0	0
Cl. botulinum type A	0	0	0	0	0	0	0	0	0	0
Cl. botulinum type B	0	+	+	0	0	0	0	0	0	0
No. 16	0	++	++	+	0	0	0	0	0	0

which also depend upon chemical structures might be correlated with heat resistance of spores. Organism No. 4, biochemically similar to but serologically distinct from PA 3679, was added to fresh pork trimmings in a concentration of 10,000 spores per gram of meat and heated at 100°C. The spores of organism No. 4 failed to survive for 20 min. Organism No. 17, biochemically distinct from but closely related serologically to PA 3679, added to fresh pork trimmings in a similar concentration, survived the heat for 9 hr. A similar heat resistance was found for spores of PA 3679. In this study, serological relationship correlated with heat resistance while biochemical reactions did not.

To determine how closely organisms No. 17 and PA 3679 were related serologically, it was necessary to use absorbed serum. Antiserum for PA 3679 was absorbed with cells of organism No. 17. A second lot of antiserum was absorbed with cells of PA 3679. These sera were then used for agglutination tests with cells of the two organisms. It was found that cells of either organism, No. 17 or PA 3679, were able to remove all agglutinins present in PA 3679 antiserum capable of agglutinating either organism. PA 3679 and organism No. 17 are seemingly identical serologically.

Of interest was the observation that cells of *Cl. sporogenes* strain Spray failed to agglutinate in PA 3679 antiserum. The latter organism was considered by Williams (1940) to be *Cl. sporogenes* strain 3679. To determine if this organism had any antigens in common with PA 3679, antiserum for PA 3679 absorbed with cells of *Cl. sporogenes* strain Spray was used. Cells of PA 3679 agglutinated in the absorbed serum yielding a ++ reaction through the 1:5120 dilution. Since the titer of the PA 3679 antiserum was not reduced by absorption with cells of *Cl. sporogenes* strain Spray, the evidence indicates that these two organisms do not have common antigens. This apparent lack of relationship is within the limits of the serological procedures used, viz. agglutination reactions at 52°C. This confirms the work of Gross et al. (1946a), who worked with non-absorbed serum and came to a similar conclusion.

Reactions of organisms in Cl. sporogenes strain No. 74 antiserum. Agglutination reactions of various putrefactive anaerobes were determined in antiserum for organism No. 74, identified biochemically as *Cl. sporogenes*. It was found that organisms No. 74, 75, 73, 7, and 4 isolated from meat and a known culture of *Cl. sporogenes* strain Spray had some antigens in common. Titers for these organisms in No. 74 antiserum were 1:1280, 1:1280, 1:320, 1:2560, 1:640, and 1:320 respectively. Similar results were obtained using strain No. 75 antiserum.

Studies with absorbed sera indicated that organisms No. 74 and 75 with the biochemical patterns of *Cl. sporogenes* were identical serologically.

Reactions of organisms in antiserum for organism No. 4. Development of agglutinins in rabbit blood for organism No. 4, biochemical pattern PA 3679, was poor, i.e., a titer of 1:320. The known culture of PA 3679 failed to agglutinate in this serum substantiating the lack of serological relationship between the two.

This study indicates some of the difficulties involved in identification of the putrefactive anaerobes. Serological results do not always confirm biochemical patterns. McCoy and McClung (1938) emphasized that serological results are not always in harmony with present taxonomic schemes. Since serological tests must eventually be combined with the biochemical reactions commonly used in order to adequately describe bacterial species, it is desirable that further studies be carried out along this line.

REFERENCES

Breed, R. S., E. G. D. Murray, and N. R. Smith. 1957. "Bergey's Manual of Determinative Bacteriology." 7th ed. Williams and Wilkins Co., Baltimore.

Burke, M. U., K. H. Steinkraus, and J. C. Ayres. 1950. Methods for determining the incidence of putrefactive anaerobic spores in meat products. *Food Technol.* **4**, 21.

Gross, C. E., C. Vinton, and C. R. Stumbo. 1946a. Bacteriological studies relating to thermal processing of canned meats. V. Characteristics of a putrefactive anaerobe used in thermal resistance studies. *Food Research* **11**, 405.

Gross, C. E., C. Vinton, and C. R. Stumbo. 1946b.

Bacteriological studies relating to thermal processing of canned meats. VI. Thermal death-time curve for spores of test putrefactive anaerobe in meat. *Food Research* **11**, 411.

McCoy, E., and L. A. McClung. 1938. Serological relations among sporeforming anaerobic bacteria. *Bacteriol. Revs.* **2**, 47.

Ogilvy, W. S., M. V. Burke, and J. C. Ayres. 1950. A practical method for controlling atmospheres for use in food research. *Food Research* **15**, 343.

Reed, O. B., and J. H. Orr. 1941. Rapid identification of gas gangrene anaerobes. *War Med.* **1**, 493.

Society of American Bacteriologists. Committee on Bacteriological Technic. 1957. "Manual of Microbiological Methods." 315 pp. McGraw-Hill Book Co., New York.

Steinkraus, K. H., and J. C. Ayres. 1963. Incidence of putrefactive anaerobic spores in meat products. *J. Food Sci.* **29**, 87.

Tanner, F. W. 1933. "Practical Bacteriology." 2nd ed. pp. 116–117. Wiley & Sons, New York.

Williams, O. B. 1940. Experimental procedure for process determination for canned foods. *Proc. Inst. Food Technologists* **1**, 323.

A Note on Orthogonal Polynomials Applied to Treatment Levels with Unequal Replications[a]

JACOB N. EISEN[b]

Human Nutrition Research Division, Agricultural Research Service
U. S. Department of Agriculture, Washington 25, D. C.

(Manuscript received February 25, 1963)

SUMMARY

This paper demonstrates how orthogonal polynomials are constructed and used when the treatment levels have unequal, rather than equal, replications. These polynomials are applied to hypothetical data on shear force of broccoli stems cooked for four cooking times with unequal replications. The variation for between cooking times (treatment levels) is broken down into independent regression components to determine the simplest regression curve appropriately representing the data. Also illustrated is how to calculate a regression equation in terms of orthogonal polynomials for treatment levels with unequal replications.

This paper is a follow up to papers on orthogonal polynomials applied to taste-panel scores for broccoli cooked for equally spaced times (Eisen *et al.*, 1960) and unequally spaced times (Eisen, 1960). In each of these applications, the treatment levels (cooking times) had the same number of replications. In another application, Robson and Atkinson (1960) constructed orthogonal polynomials to determine homogeneity of regression coefficients of gain of weight on initial weight of pigs for four lots.

Orthogonal polynomials are infrequently applied when the treatment levels have unequal, rather than equal, replications. This paper demonstrates that orthogonal polynomials can be easily constructed and advantageously used for treatment levels with unequal replications. The method of Grandage (1958) is adapted for this purpose.

METHODOLOGY

As given by Eisen (1960), suppose the regression of Y on X is represented by the equation

$$\hat{Y} = \bar{Y} + A_1P_1 + A_2P_2 \ldots + A_rP_r \qquad [1]$$

in which: P's are orthogonal polynomials such that P_1 is a function of X, P_2 of X and X^2, and in general, P_r of X, X^2 . . . and X^r. X denotes treatment level, n is the number of treatment levels, r is less than or equal to $n-1$, and A's are functions of partial regression coefficients.

For treatment levels with unequal replications, orthogonal polynomials satisfy the following two conditions:

Condition 1. The sum of the weighted values for each polynomial—P_1, P_2, . . . , *and* P_{n-1}—is zero, with the number of replications used as weights; in symbols, this condition means that

$$\Sigma wP_1 = 0,\ \Sigma wP_2 = 0,\ \ldots,\ \Sigma wP_1P_{n-1} = 0$$

in which w is the number of replications for treatment j and the sum is taken over j for $j = 1,2, \ldots n$.

Condition 2. The sum of cross products of the weighted values for any two polynomials is zero, with the number of replications used as weights (Guest, 1953); for P_1, for example, this condition means that

$$\Sigma wP_1P_2 = 0,\ \Sigma wP_1P_3 = 0, \ldots,\ \Sigma wP_1P_{n-1} = 0$$

The other polynomials P_2, $P_3, \ldots$, and P_{n-1} satisfy similar relations.

Orthogonal polynomials for treatment levels with unequal replications are constructed by supposing that Conditions 1 and 2 are satisfied by the P's in Eq. 1 as well as other polynomials differing from the P's by a constant multiplier.

For convenience in application, the values for the P polynomials are integers reduced to lowest terms and are derived from other polynomials given by

$$T_1 = k_1P_1 = a_{10} + X \qquad [2]$$

$$T_2 = k_2P_2 = a_{20} + a_{21}X + X^2 \qquad [3]$$

and so on up to T_{n-1}. In these equations, the k's are constants so chosen to yield integers for the P values and the a's are constants with subscripts designating in turn the degree of the polynomial and the power of X.

DISCUSSION

To illustrate the use of orthogonal polynomials for treatments with unequal replications, suppose broccoli stems are cooked for 5, 10, 15, and 25 min and these cooking times have 2, 3, 2, and 3 replications, respectively. For concreteness, let Y in Eq. 1 represent hypothetical shear force

[a] Adapted from an M.S. thesis, George Washington University.

[b] Present address: Food and Drug Administration, Washington, D. C.

values and X represent cooking time. The problem, then, is to obtain an analytical expression representing the effect of cooking time on shear value.

The procedure for obtaining such an expression is illustrated in the following steps:

Step 1. Since there are four cooking times, Eq. 1 contains at most three polynomials—P_1, P_2, and P_3. These orthogonal polynomials are constructed by a simple algebraic procedure that differs from the method of Grandage (1958) only by the introduction of unequal, rather than equal, replications.

For ease in computation, the cooking times are coded so that one of the coded values is zero (column 2, table 1).

Step 2. The polynomial P_1 is constructed as follows: Condition 1 is applied by supposing that $\Sigma w T_1 = 0$ (column 5). The solution of this equation yields the value of a_{10}, from which are easily calculated the values for T_1 and P_1 for cooking times of 5, 10, 15, and 25 minutes (columns 6 and 7).

Step 3. The polynomial P_2 is constructed as follows: The value of a_{21} (column 10) in the polynomial T_2 is calculated by applying Condition 2, which gives the relation $\Sigma w T_2 P_1 = 0$. Then, the value of a_{20} (column 9) is calculated by applying Condition 1, which gives the relation $\Sigma w T_2 = 0$. The values for T_2 and P_2 then easily follow (columns 11 and 12).

Step 4. The polynomial P_3 is constructed in a similar manner (not shown). The values of the a's in the polynomial

$$T_3 = a_{30} + a_{31} X + a_{32} X^2 + X^3 \qquad [4]$$

are calculated by solving the three equations $\Sigma w T_3 = 0$, $\Sigma w T_3 P_1 = 0$, $\Sigma w T_3 P_2 = 0$, derived by applying Conditions 1 and 2. The values for P_3 (Table 2) are then easily obtained.

Note that the polynomials P_1, P_2, and P_3 satisfy Conditions 1 and 2. For example, the polynomial P_2 satisfies the conditions that

$$\Sigma w P_2 = 2(152) + 1(-116) + 2(-217) + 3(82) = 0$$
$$\Sigma w P_1 P_2 = 2(-17)(152) + 1(-9)(-116) + 2(-1)(-217) + 3(15)(82) = 0$$
$$\Sigma w P_2 P_3 = 2(152)(-9) + 1(-116)(48) + 2(-217)(-18) + 3(82)(2) = 0$$

Thus, by a simple algebraic procedure, we have constructed the three orthogonal polynomials—P_1, P_2, and P_3—for four treatment levels with unequal replications.

Step 5. The appropriate regression curve representing the data is then determined by an analysis of variance (Table 3) procedure similar to that given for treatment levels with equal replications (Eisen, 1960). The only modification in this procedure is the use of replications as unequal weights in computing the sum of squares.

The noteworthy feature of this procedure is that even for treatment levels with unequal replications the method of orthogonal polynomials enables us to break down the variation for between treatments into independent regression components. Then, the appropriate regression curve representing the effect of cooking time on shear force is determined by testing the significance of each regression component [by the variance ratio (F) test comparing the mean square of a component to that for error—not shown].

Step 6. The appropriate regression equation for estimating shear force values from cooking times (only within the range of 5–25 min) is then easily calculated; the procedure is illustrated by Eisen (1960). For example, suppose that the quadratic regression component is significant but the cubic component is not. Then, Equation 1 becomes

$$\hat{Y} = \bar{Y} + A_1 P_1 + A_2 P_2 \qquad [5]$$

This equation is derived by substituting the values of $\bar{Y} = 51$ (the mean shear force value for the four cooking times), A_1 and A_2, and the expressions for P_1 and P_2 in terms of X (Table 2). These expressions are derived from Table 1. For example, the expression

$$P_1 = 8Z - 17 = 1.6X - 25$$

is derived from columns 4–7 in that table, which give

$$T_1 = a_{10} + Z = -17/8 + Z$$

and

$$8T_1 = 8Z - 17 = 1.6X - 25 = P_1$$

The noteworthy feature of using orthogonal polynomials to derive the appropriate regression equation for treatment levels with unequal replications is that each successive

Table 1. Method of constructing orthogonal polynomials, P_1 and P_2, for four treatment levels with unequal replications.

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
X	$Z=(X/5)-1$	w	$T_1=a_{10}+Z$	wT_1	T_1	$8T_1=P_1$	$T_2=a_{20}+a_{21}Z+Z^2$	wT_2	wT_2P_1	T_2	$(167/2)T_2=P_2$
5	0	2	a_{10}	$2a_{10}$	$-17/8$	-17	a_{20}	$2a_{20}$	$-34a_{20}$	304/167	152
10	1	1	$a_{10}+1$	$a_{10}+1$	$-9/8$	-9	$a_{20}+a_{21}+1$	$a_{20}+a_{21}+1$	$-9a_{20}-9a_{21}-9$	$-232/167$	-116
15	2	2	$a_{10}+2$	$2a_{10}+4$	$-1/8$	-1	$a_{20}+2a_{21}+4$	$2a_{20}+4a_{21}+8$	$-2a_{20}-4a_{21}-8$	$-434/167$	-217
25	4	3	$a_{10}+4$	$3a_{10}+12$	15/8	15	$a_{20}+4a_{21}+16$	$3a_{20}+12a_{21}+48$	$45a_{20}+180a_{21}+720$	164/167	82
Sum				$\Sigma wT_1=$ $8a_{10}+17=0$				$\Sigma wT_2=8a_{20}+17a_{21}$ $+57=0$	$\Sigma wT_2P_1=$ $167a_{21}+703=0$		
				$a_{10}=-17/8$				Since $a_{21}=$ $-703/167$ (column 10), $a_{20}=$ $\frac{-57-17(-703/167)}{8}$ $=304/167$	$a_{21}=-703/167$		

Explanation of columns

Column

1. Treatment level, actual values
2. Treatment level, coded values
3. Number of replications for each treatment level
4. General equation for T_1 in terms of coded values for treatment levels
5. Multiply column (4) by column (3)
6. Substitute the value for a_{10} in column (4)
7. Multiply column (6) by 8
8. General equation for T_2 in terms of coded values for treatment levels
9. Multiply column (8) by column (3)
10. Multiply column (9) by column (7)
11. Substitute the values of a_{20} and a_{21} in column 8
12. Multiply column (11) by 167/2

Table 2. Polynomial values and polynomial expressions for four cooking times with unequal replications.

Cooking time X (min)	$Z=(X/5)-1$	Shear value (mean) $\bar{Y}$ (lb force)	Number of replications (w)	Polynomial values P_1[a]	P_2[b]	P_3[c]
5	0	150	2	−17	152	−9
10	1	48	1	−9	−116	48
15	2	18	2	−1	−217	−18
25	4	8	3	15	82	2
ΣwYP_i				−5208	34188	−996
ΣwP_i^2				1336	174014	3126
$A_i=\Sigma wYP_i/\Sigma wP_i^2$				−3.8982	0.1965	−0.3186

[a] $P_1 = 8Z - 17 = 1.6X - 25$
[b] $P_2 = 83.5Z^2 - 351.5Z + 152 = 3.34X^2 - 103.7X + 587$
[c] $P_3 = 1/24\ (521Z^3 - 3039Z^2 + 3886Z - 216) = 1/3000\ (521X^3 - 23010X^2 + 288175X - 957750)$

Table 3. Analysis of variance: breakdown of "between treatments (cooking times)" by orthogonal polynomials into independent components of regression of shear force value on cooking times.

Source of variation	Sum of squares[a]	Degrees of freedom	Mean square
Between cooking times	27336	3	9112
Linear component	20301.84	1	20301.84
Quadratic component	6716.81	1	6716.81
Cubic component	317.34	1	317.34

See Table 2 for values of ΣwYP_i and ΣwP_i^2.
[a] Between cooking times:
$\Sigma wY^2-(\Sigma wY)^2/\Sigma w = 48144 - 20808 = 27336$
Linear component:
$(\Sigma wYP_1)^2/\Sigma wP_1^2 = (-5208)^2/1336 = 20301.84$
Quadratic component:
$(\Sigma wYP_2)^2/\Sigma wP_2^2 = (34188)^2/174014 = 6716.81$
Cubic component:
$(\Sigma wYP_3)^2/\Sigma wP_3^2 = (-996)^2/3126 = 317.34$
The sums of squares for these three components equal, except for rounding, the sum of squares for "between cooking times."

higher-degree equation is formed by the addition of lower-degree terms already computed. For example, the quadratic (Eq. 5) is formed by adding A_2P_2 to the linear equation $\hat{Y} = \bar{Y} + A_1P_1$.

CONCLUSIONS

For a small number of treatment levels with unequal replications, orthogonal polynomials can be conveniently constructed and used advantageously as outlined herein. The algebraic computations are particularly simplified when only the linear and quadratic components are desired or when the sum of squares for the highest degree component is calculated by subtraction. When this method is inconvenient to use—as in constructing all the orthogonal polynomials for six or more treatment levels with unequal replications—the matrix inversion method (Wishart and Metakides, 1953) may be preferred.

There are numerous areas in which orthogonal polynomials can be advantageously applied for treatment levels with unequal numbers of replications. In addition to cooking experiments, as in this example, some of these areas of application are: Processing experiments for determining effect of storage times or temperature on vitamin content of foods with unequal replications for storage time or temperature levels; longevity experiments in which the treatment levels are age of animal survival; and pharmaceutical experiments to determine effect of a drug administered in various amounts to different numbers of subjects.

REFERENCES

Eisen, J. N., A. B. Parks, and E. H. Dawson, 1960. Application of orthogonal polynomials to data on palatability. *Food Technol.* **14**, 239.

Eisen, J. N. 1960. A note on the application of orthogonal polynomials to data on palatability when the levels of a treatment are unequally spaced. *Food Technol.* **14**, 663.

Grandage, A. 1958. Orthogonal coefficients for unequal intervals. *Biometrics* **14**, Query 130, 287.

Guest, P. G. 1953. The Doolittle method and the fitting of polynomials to weighted data. *Biometrika* **40**, 229.

Robson, D. S., and G. F. Atkinson. 1960. Individual degrees of freedom for testing homogeneity of regression coefficients in a one-way analysis of variance. *Biometrics* **16**, 593.

Wishart, J., and T. Metakides. 1953. Orthogonal polynomial fitting. *Biometrika* **40**, 361.

Determination of Contribution Coefficients in Sensory Scoring of Over-All Quality

NINA BARYLKO-PIKIELNA AND KAZIMIERZ METELSKI
Meat Research Institute, Warsaw, Poland

(Manuscript received February 18, 1963)

SUMMARY

A method for mathematical determination of contribution coefficients for the sensory scoring of food-stuffs was presented and a regression equation for the over-all product quality on the basis of canned ham specified.

Using the afore-mentioned method, quality factors decisive for the over-all quality of the evaluated product may be also determined.

The scoring of the organoleptic quality of foodstuffs is one of the quality evaluation methods most used in research and industrial quality control. It is based on evaluation of quality factors such as color, odor, juiciness, tenderness, palatability, according to an adopted scale.

Many scoring scales are used, of varying construction and with different scoring ranges (Wagner, 1949; St. Kaniuga, 1953). Comparisons based on the scoring evaluation theory have shown that the most appropriate are scales with a small and identical number of scores for each factor (Wagner, 1949; St. Kaniuga, 1953; Tilgner, 1957; Plank, 1948; Tilgner, 1959).

Using scores (and corresponding descriptive terms) for individual quality factors over-all product quality is expressed as a combined score. Such score is necessary in many cases, especially in quality control, because it is more comprehensible and easier to interpret than tabulation of the scores for several individual quality factors.

The combined score should illustrate the over-all quality of the product in the best possible way. There are different approaches to the determination of this over-all quality measurement, based on the evaluation of the partial qualities (scores for the individual quality factors). It may be expressed by: 1) the sum of scores (or their weighted mean); 2) the quality class determined by the critical limits, i.e., established tolerances for the original quality factors (those that have decisive importance for over-all quality); 3) an index of the general quality (computed with arbitrary contribution coefficients for individual quality factors). Since the quality factors contribute at different levels to the over-all quality, a mere addition of the partial scores clouds the assessment of over-all quality and therefore is only of relative value.

The method of critical limits leads to a more correct assessment of over-all quality, but its application is confined in principle to quality assessment from the point of view of commercial usefulness. Contribution coefficients allow a differentiation of the importance of the individual quality factors as to the degree to which they influence over-all quality, but only on condition that they are properly determined. At present, establishment of these coefficients for each product by a panel or even single professional experts is to a high degree arbitrary (Kaniuga, 1953; Plank, 1948; Tilgner, 1957; Ehrenberg, 1953).

An experiment has been carried out to assess the quality of the product as a function of the partial scores. The example used was the scoring of pasteurized hams.

EXPERIMENTAL METHODS

Several pasteurized ham samples were presented to an experienced laboratory panel made up of 8 persons. Quality scoring was done on color, slice-binding, odor, juiciness, tenderness, flavor, and saltiness using a 5-point scale (Tilgner, 1950, 1957).

The same panel also was asked to give an over-all quality score. Altogether, 55 evaluations were made.

The initial data were used for the calculation of contribution coefficients. In calculating these coefficients it was assumed that the greater the score for a given quality factor (x_i) the more it will influence the over-all quality and the more consistent will be its score with the over-all one.

Since individual quality factors are usually correlated with one another, the dependence between scores for given factors and the general evaluation (y) was studied independently of the influence of other factors. Thus, the problem was looked upon as a partial regression of the scores for the individual quality factors with respect to the general score. The partial coefficients a and γ (Table 1), calculated by means of the inverse of the matrix of the simple correlation coefficients, are then the contribution coefficients.

The degree of interdependence between the factors x_i and the general score y (i.e., the contribution of the individual factors) was established by:

1) *Partial correlation*—r_{ij}. The numerical values are given in column 4 of Table 1. Column 5 gives their relative magnitude.

2) *Sum of squares* attributable to the individual regression coefficients are s^2a_{iy}. The advantage of using this is that the sums of squares for the partial regression coefficients are additive. Column 7 of Table 1 gives the percent share (s^2a_{iy} divided by Σs^2a_{iy} times 100) of the regression ascribable to each factor.

Thus it is obvious that *ca.* 43% of the variance is ascribable to flavor. Column 8 gives the relative position according to the sum of squares s^2a_{iy} attributable to the respective regression coefficients.

3) *Coefficient* β (i.e., coefficient of partial regression) expressed as a standard deviation. The values are shown in column 9. Their order is in essential agreement with that for partial correlation coefficients.

This approach was suggested by Ezekiel and Fox (1949) and Steel and Torrie (1960).

The negative values a_{3y} and r_{3y} require explanation. This is explained by the high correlation between this factor and the others which have a high correlation coefficient with the over-all score. Also by the fact that, according to Polish export requirements, the highest score is given to the leanest ham and the lowest to the fattest. This does not agree with the preference expressed by the panel used (computed on the basis of the obtained experimental results).

The regression equation is of the form

$$Y = -0.58 + \Sigma a_{iy}x_i$$

Table 1. Statistical relationship in quality scores.

Quality: 1	2	3	4	5	6	7	8	9
Factor no. (i)	Type	a_{iy}	r_{ij} Value	Order	S^2a_{ij}	$\frac{s^2a_{iy} \times 100}{\Sigma s^2a_{iy}}$	Order	β
1	color	.0746	.2013	6	.0869	5.28	5	.1052
2	fatness	−.2182 [a]	−.6093	3	.2378	1.44	8	−.3329
3	slice binding	−.0299	.0846	8	.2586	1.57	7	−.0402
4	odor	.3416 [a]	.6168	2	3.9626	24.07	2	.3678
5	juiciness	.2562 [a]	.5907	4	2.1495	13.05	3	.3306
6	tenderness	−.0553	.1205	7	.4336	2.63	6	−.0583
7	flavor	.5514 [a]	.7974	1	7.0745	42.97	1	.5809
8	saltiness	.1779 [a]	.4745	5	1.4801	8.99	4	.2340
					16.4657	100.00		

[a] Significance level $\alpha = 0.05$.

It is noted that this plane does not pass through the origin of the coordinates (y, x_2, . . . x_9). If the regression plane is forced to pass through origin then the calculated values of the a_{iy} are unreasonable and the plane does not pass through the point (y,x_2, . . . x_9).

For theoretical reasons this variant was calculated, too. Calculated coefficients values (a_{iy}) are unreasonable, and this confirms

the thesis: the supposition is unacceptable that the hyperplane of the regression passes the beginning of the coordinates.

The above mathematical interpretation of the scores for ham quality indicates that it is possible to: 1) state objectively what quality factors essentially influence the general score of the product; 2) state the contribution of each factor in the general score; 3) determine the regression equation expressing the over-all quality based on sums for the individual quality factors; 4) eliminate the arbitrarily fixed contribution coefficients employed hitherto in determining the over-all quality of the product. It must be emphasized that the contribution coefficients will vary in various countries with consumer preference and acceptability with regard to the individual quality factors. For instance in a society that is above all accustomed to visual quality evaluation such components as color and other optical impressions will presumably have a bigger share.

The obtained sequence of contribution must be looked upon as specific for Polish ham and Polish scoring panels. But the above approach may—in our opinion—be also generally used for the objective determination of critical factors and the method of critical limits.

REFERENCES

Eherenberg, A. S. C. 1953. On Plank's rational method of grading food quality. *Food Technol.* **7**, 188.

Ezekiel, M., and K. A. Fox. 1959. "Methods of Correlation and Regression Analysis." 3d ed. New York.

Goulden, C. H. 1956. "Methods of Statistical Analysis." New York.

Plank, R. 1948. A rational method for grading food quality. *Food Technol.* **7**, 241.

St. Kaniuga. 1953. Systemy punktowej oceny organoleptycznej produktów zywnosciowych. *Przemyst Rolny i Spozywczy.* **7**. 137.

Steel, R. G. D. and J. H. Torrie. 1960. "Principles and Procedures of Statistics," New York.

Tilgner, D. J. 1957. "Analiza Organolept Zywnosci." Warszawa.

Tilgner, D. J., and Z. Osinska. 1950. Niektóre skladniki jakosci szynek konserwowych. *Przemyst Rolny i Spozywczy.* **4**, 131.

Tilgner, D. J., and N. Barylko-Pikielna. 1959. Wady róznych metod punktowych a klasyfikowanie jakosci. *Przemyst Spozywczy.* **13**, 429.

Wagner, G. 1949. Zur Theorie der Bewertungsschema für Lebensmittel. *Deut. Lebensm.-Rundschau.* **45**, 145.

Sensory Comparisons: The 2-Stage Triangle Test with Sample Variability[a]

N. T. GRIDGEMAN
Division of Applied Biology, National Research Council, Ottawa 2, Canada

(Manuscript received May 27, 1963)

SUMMARY

The first stage of this test is the attempt to identify the odd member of a coded AAB or ABB triad formed of samples of two materials, A and B, that are to be sensorily compared. The second stage is a ranking decision on the selected item with regard to the specific sensory quality or to preference. When the materials, and consequently the samples, are heterogeneous, the sameness of the duplicates cannot he guaranteed, and a sampling distribution must be postulated before valid inferences can be made. A probabilistic model of the test is constructed. Experimental data on tenderness of poultry meats are shown to fit this model.

If two similar materials are to be examined for a possible sensory difference in, for example, flavor, the two-stage triangle test may be used. A judge, presented with a coded triad of samples (one of one material, and two of the other), is asked, first, "Which of the three samples is odd, with regard to flavor?" and second, "Do you think that the sample you have so chosen is more or less flavorsome than the other two?" Such an approach seems intuitively rewarding; in the first stage, discrimination only is at issue; in the second stage, supplementary directional information is being elicited. (There is evidence and argument for a specific faculty of discrimination *per se* in this kind of work, see Gridgeman (1959), although it need not be adduced for the present purpose.) In some circumstances the second stage may be hedonic, with the question, "Do you like the sample you have chosen more or less than the other two?" and then the two stages are concerned with different aspects of the material-judge sensory reaction.

The theory of this type of test has been neglected, and what follows is a first, and not necessarily the best, approach. It deals with a specialized form of the test, *viz.*, when the materials are heterogeneous. To this end the symbols $\overline{A}$ and $\overline{B}$ are used to distinguish the actual materials from particular samples, A and B, respectively. Thus, $\overline{A}$ might be cabbages grown under a certain horticultural regimen, and A would then be one cabbage, or even part of a cabbage, from the lot.

Let us begin by recalling the general circumstances in which an ordinary triangle test, rather than straight paired comparison, is chosen as an experimental design. They are: 1) It is more immediately important to ascertain whether a difference exists than to collect sensory evidence of its character and direction (which aspects may be obvious from extraneous considerations). The test panel's task is thus kept simple, and a minimum of training or indoctrination is called for. 2) The essence of the possible difference is hard to define objectively and unambiguously. Various kinds of non-quantitative differences in appearance and color fall into this class. Again, in checking, say, freshness in meats, the investigator may find that paired comparison is bedeviled by the fact that certain off-flavors are attractive and palatable to some panel members but offensive to others.

The greater specificity needed in paired comparison must be emphasized. You cannot give a judge one coded sample each of A and B and simply ask "Do they differ?" for there is no built-in protection against psychosensory bias—consciously or other-

[a] N.R.C. No. 7698.

wise he will be tempted to answer in the affirmative because he expects a difference and hopes that his sensory acuity is good enough to detect it. So, although, generally speaking, paired comparisons are rather more efficient than triangle tests (Gridgeman, 1955; Ura, 1960), situations arise in which the latter are more appropriate. We now go on to consider circumstances in which a triangle test can be supplemented by some simple "either/or" question.

THEORY

Our *test instrument* is taken to be a panel of scrupulous, reasonably concordant, and reasonably sensitive judges. This is a conceptual simplification, of course, for some of the observed variability, here wholly attributed to material heterogeneity, will in fact stem from judge variability, but no serious distortion of the picture is to be expected.

The *materials*, $\bar{A}$ and $\bar{B}$, as already intimated, are assumed not to be easy to sample. To exemplify: imagine the experimental treatments that distinguish two meats $\bar{A}$ and $\bar{B}$ to be such as can be used only on whole carcasses (perhaps even on the living animal) or large cuts. Because the normal differences between carcasses are nontrivial, wide sampling is necessary. Thus, two ostensibly identical samples of the same treatment, in a particular trial, may differ between themselves—and the difference could be as great as, or even greater than, that between the two treatments. In the long run these intra-treatment differences will cancel out, but this does not erase the statistical problem of the handling of the panel's decisions—when they are not unanimous.

It is not difficult to think of similar situations in other branches of food research. For instance, in an investigation of egg-treatment differences, the variability among samples in any one treatment has to be taken into account. Other small items of natural produce, such as fruits and vegetables, may also be cited.

A triangle trial has two balancing forms, AAB and ABB; and for reasons of symmetry (see Appendix I) these must be equally represented; moreover, every judge should carry out the same number of trials. The 2-stage version of the test has four possible outcomes, depending upon whether, in the first stage, the correct choice of the odd member has been made, and upon how, in the second stage, this chosen member is ranked (e.g., more or less tender; more or less liked). The totality of results from N trials can be entered in a 2×2 frequency table as follows (here R is the number of results of each kind, and the characterizing significance of the subscripts will be obvious):

		Stage 2: Ranking (decoded) of selection from Stage 1		
Stage 1		A>B	A<B	Sums
Selection of putative odd sample	correct	R_{ca}	R_{cb}	R_c
	incorrect	R_{ia}	R_{ib}	R_i
	Sums	R_a	R_b	N

Plainly, if the distinctions between A and B in all trials were clear-cut, the result table would read

0	N	N			N	0	N
0	0	0	or		0	0	0
0	N	N			N	0	N

and if the distinctions were imperceptible (or averaged out to zero) the possible results would randomly fluctuate about the expectations

$N/6$	$N/6$	$N/3$
$N/3$	$N/3$	$2N/3$
$N/2$	$N/2$	N

provided that the judges were obligated to make the double decisions on every occasion, regardless. There is also an intermediate expectation, viz.,

$N/2$	$N/2$	N
0	0	0
$N/2$	$N/2$	N

which could happen if there was full agreement about the identity of the odd member (i.e., perfect discrimination) allied with maximum disunity on the second-stage, ranking, decision. There just might be a tendency in this direction if the second stage turned on the intensity of a characteristic, but there could very easily be a strong tendency if the second stage is hedonic.

These "null" expectations suggest the use of the two observed sets of marginal data (R_c versus R_i, and R_a versus R_b) for binomial significance tests. Yet second thoughts may raise doubts as to whether this is the proper thing to do, for the straight comparison of R_a and R_b means that we have in effect given equal weight to tenderness judgments on the correct selections (of the odd sample) and on the incorrect ones. Let us then consider the sort of distribution of results that may be expected in the non-null case, i.e., when $\bar{A}$ and $\bar{B}$ exhibit perceptible (but not obvious) differences, under a few assumptions about sample variability.

Suppose that all test samples, A of $\bar{A}$ or B of $\bar{B}$, are drawn from a symmetrical distribution centering on some mean value on a psychosensory

intensity scale of the property at issue—here tenderness. We might for instance conceive of a 10-point scale on which random lots of 100 samples of A and B are spread in this fashion

Arbitrary degree of intensity (of tenderness)	Frequency distribution of 100 samples A of $\overline{A}$	Frequency distribution of 100 samples of B of $\overline{B}$
1	4	.....
2	8	.....
3	22	1
4	37	10
5	17	19
6	9	40
7	2	20
8	1	8
9	.....	2
10	.....	.....

Note that the distributions are roughly similar. That is to say, although the means differ (approximately 4.0 for $\overline{A}$ and 6.0 for $\overline{B}$), the d spersions are much the same. Now, if two samples of $\overline{A}$ and one sample of $\overline{B}$ are picked at random from such lots to constitute a triangle trial, the two A's could by chance differ more from each other than either from the B, in which case one of the A's would be the truly odd member. Which in turn means that the labels "correct" and "incorrect" in the primary 2×2 result table are equivocal; *they apply to the materials themselves, not to the test samples.* This distinction must be kept firmly in mind, for it means that a judge can make what appears to be a misidentification in Stage 1, and yet make a correct (or at least a meaningful) ranking decision in Stage 2.

Consider all possible locations of the three samples along the intensity continuum. Since the two samples of A are in practice coded differently, they had better be distinguished in some way here; so we shall write A_1 and A_2. We shall also (ignoring hedonic choices for the moment) use two ranking signs: ">" to mean "is stronger than" or "is tenderer than" or some such, and ">>" to mean the same only more so. Thus "$A_1 > A_2 >> B$" means that a triad in which the sample A_1 is the "strongest" in regard to the property at issue, and B is the "weakest," but that the difference between A_1 and A_1 is less than that between A_2 and B. Now if, for simplicity of presentation, we ignore the cases in which one or more samples happen to be identically located, or in which the three samples happen to be equally spaced along the continuum, the possible dispositions for an AAB triad can be grouped into 12 classes, each associated with a particular one of the four possible decisions. They are shown in Table 1. Similarly, but complementarily, another set exists for the ABB triad.

The probabilities of the four kinds of decision, *ca, cb, ia,* and *ib,* depend on the frequency distribution of the samples along the sensory intensity continuum, and on the interval between the two means, $\overline{A}$ and $\overline{B}$ (a distance measurable in units of the common standard deviation of the distributions). These frequencies are not ordinarily observable, but we can make a simple distribution assumption and then go on to the test fit of the decision frequencies in a well-replicated experiment.

Strictly, symmetry is the only characteristic of the distribution needed to justify the pooling of the ranking decisions (A versus B) from both the "correct" and "incorrect" stage-1 groupings. To construct a model, however, a specific distribution must be used, and the natural choice is the normal (Gaussian) function. The four p's for any $d = |\overline{A} - \overline{B}|$, can then be calculated. Ura's work (1960) on the analysis of sensory-response fluctuations in "straight" pair and triangle tests provides a mathematical basis for such calculations—and it also indicates that the resultant statistical model is robust. More detail is given in Appendix II; the over-all results are shown in the form of probability curves in Fig. 1.

Table 1. The possible samplings to form an AAB triad, and the resultant decisions.

	The decisions		
	Stage 1	Stage 2	
Relative disposition of samples in regard to intensity of factor at issue (e.g., tenderness)	Selection of sample oddity in terms of material oddity	Material (decoded) judged to have the greater intensity	Decision reference (decoded)
$A_1 > A_2 >> B$	correct	$\overline{A}$	*ca*
$A_2 > A_1 >> B$	correct	$\overline{A}$	*ca*
$B >> A_1 > A_2$	correct	$\overline{B}$	*cb*
$B >> A_2 > A_1$	correct	$\overline{B}$	*cb*
$A_1 > B >> A_2$	incorrect	$\overline{B}$	*ib*
$A_2 > B >> A_1$	incorrect	$\overline{B}$	*ib*
$B > A_1 >> A_2$	incorrect	$\overline{B}$	*ib*
$B > A_2 >> A_1$	incorrect	$\overline{B}$	*ib*
$A_1 >> A_2 > B$	incorrect	$\overline{A}$	*ia*
$A_1 >> B > A_2$	incorrect	$\overline{A}$	*ia*
$A_2 >> A_1 > B$	incorrect	$\overline{A}$	*ia*
$A_2 >> B > A_1$	incorrect	$\overline{A}$	*ia*

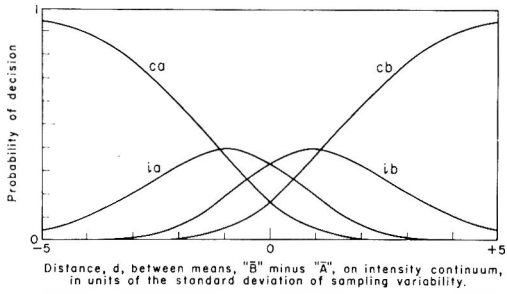


Fig. 1. Probabilities of decision as conditioned by difference between sample means.

Key to probability curves:

1st stage { *c* correct selection of putative oddity; *i* incorrect selection of putative oddity

2nd stage { *a* A judged stronger than B; *b* B judged stronger than A

Some results. Seven experiments on tenderness of poultry meats were carried out over a period of months. Each was a comparison of two treatments. The subjective appraisals were done by means of two-stage triangle tests.

The essential information is assembled in Table 2. For convenience, the experiments are arranged in order of the stage-two (ranking) probabilities; that is to say, of the strength of evidence that the two materials are not equally tender (and the codings have been switched, where necessary, so that the material earning the majority vote in each experiment is always labeled "$\overline{B}$"). Observe that the correlation between the ability to discriminate and the ability to rank (in terms of tenderness) is not strong. The rankings made on the nominally incorrect selections (of the oddity) in the first stages can be seen to be about as "good" as those made on the nominally correct selections—and this is consonant with the proposed model.

The plausibility of the model is further supported by goodness-of-fit tests. To this end, estimates of the distance $d = \overline{B} - \overline{A}$ (on the tenderness continuum, in units of the standard deviation of the sample distributions) were made by visual spotting of the maxima of the likelihood curves. The estimates are given in Table 2. The expected frequencies in the four categories were then computed and checked against the observed values. The resultant over-all goodness-of-fit test yielded a χ^2 of 17.1 for 21 degrees of freedom, which is plainly satisfactory.

DISCUSSION

Other ways of handling the data merit consideration. For instance, a general test for the absence of either discrimination or rankability might be attractive, and perhaps

Table 2. Results of some 2-stage triangle tests on relative tenderness of poultry meats.

	Experiment reference:	I	II	III	IV	V	VI	VII
	No. of trials per experiment:	36	36	36	36	36	36	30
Raw data	Frequencies R_a: R_{ca}	9	7	2	6	4	5	3
	Frequencies R_a: R_{ia}	9	10	14	8	7	6	5
	Frequencies R_b: R_{cb}	6	8	9	9	5	10	9
	Frequencies R_b: R_{ib}	12	11	11	13	20	15	13
Inference concerning discriminability (stage 1)	Probability (binomial, 1-tail) that frequency of correct selection of odd sample is fortuitous	0.18	0.18	0.68	0.18	0.89	0.18	0.26
Inference concerning ranking (stage 2)	Probability (binomial, 2-tail) that the observed split, $R_a : R_b$, could be fortuitous	1.00	0.87	0.62	0.24	0.03	0.03	0.02
Estimated distance, *d*, between $\overline{A}$ and $\overline{B}$ on the tenderness continuum, in units of the standard deviation of samples		0.05	0.08	0.42	0.32	0.36	0.60	0.82
Test of over-all goodness-of-fit of the data to proposed model		$\chi^2_{21} = 17.14$; p = 0.71						

the best method is to calculate the joint probability for the two null hypotheses from the single binomial probabilities of the marginal splits (i.e., the probabilities shown in Table 2). Twice the natural logarithm of the product of the reciprocals of these two probabilities gives χ^2 for 4 degrees of freedom, and the corresponding tabular probability is what is required (Fisher, 1946).

In formulating the model we have assumed that A_1 and A_2, the twin samples of material $\bar{A}$, have zero correlation. This is in fact one extreme of a range of possible correlation, the other being unity, when the twin samples are identical. As the correlation diminishes, the chance of picking out a sample oddity that is also the material oddity, will also diminish. Thus the situation we have been examining is, in one sense, the hardest.

Whether or not the present picture of the 2-stage triangle test is acceptable (and further work, especially with real $\bar{A}$, $\bar{B}$ differences, is needed to test its acceptability), the question arises as to the circumstances that justify its employment. More experience and thought are needed here. When preference is at issue in the second stage, justification is of course clear enough, and it has an important place in consumer survey work.

ACKNOWLEDGMENT

Thanks to Mr. L. van den Berg, of this Division, for drawing attention to the problem, for some realistic discussion of it, and for allowing his experimental data to be used here.

APPENDIX I

Unless the triangles in any one experiment are equally divided between the two forms AAB and ABB, scotching of psychosensory bias cannot be guaranteed. For, suppose AAB only is used and that the panel cannot really distinguish between the 2 materials yet has a predilection to declare that the putative odd sample is the stronger; then the limiting expectations would be

0	$N/3$	$N/3$
$2N/3$	0	$2N/3$
$2N/3$	$N/3$	N

which is maximally biased. But, with as many ABB's as AAB's the expectations are unbiased, and wholly independent of the judges' idiosyncrasies.

The two forms should be allocated at random among the trials, even when two or more trials per judge are carried out. At first thought it seems proper that, if, say, each judge carries out two trials, he should do one of each form, but this arrangement is open to psychosensory bias, for a judge who expected such an alternation would tend to come up with complementary answers for his pair of trials.

Allocations should in fact not only be random, but the judges should be made aware that this is the case.

These details of experimental design are not of great consequence, but their application involves no extra work, and they do ensure the best conditions for the operation of the laws of probability.

APPENDIX II

The statistical model rests on the following assumptions:

i) Each member of the triad AA'B is a random sample from a population of actually or hypothetically available items whose relevant intensities (i.e., of the sensory property, for example, tenderness or flavor) are symmetrically distributed about a mean point on a psychosensory intensity continuum.

ii) There are two such distributions, one centering on $\bar{A}$ (from which samples A and A' are drawn), and another on $\bar{B}$ (from which sample B is drawn). They are identical in shape and, in particular, have a common standard deviation, which is used as the unit in which the distance between $d = \bar{B} - \bar{A}$ is measured. (The object of the test is of course to ascertain whether d is significantly different from zero.)

iii) The relative disposition of the 3 samples on the continuum governs the answers at each of the two stages of the trial, the most remote sample being judged odd.

These assumptions are sufficient to establish, in general terms of multiple integration over the 3-dimensional sample space, the four required probabilities: $P(ca)$, of correct selection, with "A" judged the stronger; $P(ib)$, of incorrect selection, with "B" judged the stronger; and $P(ia)$ and $P(cb)$, homologously. Each probability is to be thought of as the limiting frequency, as the size of the experiment becomes infinitely large, of the corresponding decision frequency. Thus, for instance, $P(ca)$ is the ultimate value of R_{ca}/N, as N increases.

Any pair sum of these 4 probabilities will be the parameter of a Bernoulli binomial frequency distribution for an N-sized sample. However, to quantify the probabilities we need a fourth assumption, defining a plausible distribution, and it is natural and convenient to settle for this:

iv) The cumulative distribution function is Gaussian. That is to say,

$$F(x) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{x} \exp \frac{-u^2}{2} du$$

and we conceive x to be measured in units of the standard deviation along the abscissa, which is also the sensory intensity continuum.

On these assumptions the defining equations can be shown to be:

Numerical evaluation of the P's, for $d = 0(0.2)5$, furnished the data for the plots in Fig. 1.

Although the Gaussian function is first choice, it may well be asked how crucial this, or any other function, is to the model. In fact, much latitude is pemissible, for Ura (1960) has shown that, for straight pair tests, triangle tests, and duo-trio tests, even the rather unlikely rectangle distribution yields probability curves close to those based on the normal distribution.

	Value when d is		
	Very large and negative	Zero	Very large and positive
$P(ca) = 2 \int_0^{\infty} F(-\sqrt{3}x - \sqrt{2/3}d)\,dF(x)$	1	1/6	0
$P(ia) = 1 - F(d/\sqrt{2}) - P(ca)$	0	1/3	0
$P(cb) = 2 \int_0^{\infty} F(-\sqrt{3}x + \sqrt{2/3}d)\,dF(x)$	0	1/6	1
$P(ib) = F(d/\sqrt{2}) - P(cb)$	0	1/3	0

REFERENCES

Fisher, R. A. 1946. "Statistical Methods for Research Workers." 10th ed. Oliver and Boyd, London.

Gridgeman, N. T. 1955. Taste comparisons: Two samples or three? *Food Technol.* **9**, 148.

Gridgeman, N. T. 1959. Sensory item sorting. *Biometrics* **15**, 298.

Ura, S. 1960. Pair, triangle, and duo-trio tests. *Repts. Statist. Appl. Research Union, Japan Scientists and Engs.*, **7**, 107.

The Nutritive Value of Eight Varieties of Cowpea (Vigna sinensis)[a, b]

LUIZ G. ELIAS, ROBERTO COLINDRES,[c] AND RICARDO BRESSANI
Institute of Nutrition of Central America and Panama (INCAP), Guatemala, C. A.

(Manuscript received May 31, 1963)

SUMMARY

Samples of eight varieties of cowpea, both raw and cooked, were analyzed for their chemical composition and amino acid content by microbiological methods and were also subjected to biological trials with rats in order to determine their nutritive value. Cooking caused losses as follows: 9.2% nitrogen, 17.4% crude fiber, 62% thiamine, 52% riboflavin, and 45% niacin. With the possible exception of tryptophan, the variation for amino acids was small among all the samples, both cooked and raw, and there was no difference in ether extract and ash. The animal experiments showed marked differences in protein value among the eight samples, even though essential amino acid composition indicated only slight differences. These differences are probably due to variation in amino acid availability. The protein efficiency ratio was higher in the cowpea samples than in the beans. Since cowpeas have a higher nutritive value than common beans, as confirmed in this study, and can be grown under many environmental conditions with higher yields, their use in human feeding should be recommended in developing areas of the world having protein in low quantity and quality.

Dietary surveys in the Central American countries have shown that corn (*Zea mays*) and beans (*Phaseolus vulgaris*) are the two most important sources of protein in rural diets (Flores, 1961). From the nutritional point of view, cereal grains not only are low in total protein but are deficient in some of the essential amino acids, particularly lysine. Leguminous seeds, such as cowpeas (*Vigna sinensis*), could supply the limiting amino acid in corn protein as well as replace black beans, since the two are very much alike in appearance, texture, and flavor; they could also be a better supplement for cereal-based diets.

The essential amino acid content of the cowpea has been studied by several investigators (Bressani *et al.*, 1961; Busson *et al.*, 1959; Jaffé, 1949; Orr and Watt, 1957) and the results indicated that methionine is the most limiting amino acid. The cowpea, like other leguminous seeds, contains a trypsin inhibitor that has been the object of several studies. According to Sohonie and Bhandarker (1954), the cowpea trypsin inhibitor can be destroyed by heating 1 hr at 100°C. Jaffé (1950a), from studies of the relationship between the digestibility of cowpeas and its trypsin inhibitor, reported that heating in the autoclave was sufficient to destroy the inhibitor, but that this treatment did not improve the coefficient of digestibility. Similar studies have been reported by Borchers and Ackerson (1950).

Biological studies have also been undertaken by several investigators (Borchers and Ackerson, 1950; Chavez *et al.*, 1952; Finks *et al.*, 1922; Jaffé, 1949; Sherwood *et al.*, 1954), who reported that cowpea protein is limited in methionine but is a good source of lysine. The present study was designed to obtain additional chemical and biological data on the cowpea.

MATERIALS AND METHODS

Samples and analytical procedures. Eight sample varieties of cowpea from the United States that had been introduced into Guatemala in 1959, were used: 1) Calico Crowder, 2) Brown Eye Crowder,

[a] Investigation supported by Grant RF-60180 from the Rockefeller Foundation.

[b] INCAP Publication I-286.

[c] From a thesis by R. Colindres for a degree in Chemistry and Pharmacy from the University of Honduras, Tegucigalpa, Honduras.

3) Black Eye Peas, 4) Blue Goose, 5) Bunch Purple Hull, 6) Black Crowder, 7) Lady Peas, and 8) Dixie Lee Peas. The samples were certified seed obtained from a commercial store. They were harvested from experimental plots in the lowlands of Guatemala, of highly fertile soils with high rainfall. Ten pounds of each variety were stored at 4°C. Each sample was divided into two parts; 450 g were used for chemical analysis of the raw material, and the remaining quantity was cooked and used in biological trials with rats and for chemical analysis after cooking. About 7 lb of each variety were first soaked in sufficient water for about 2 hr; they were then covered completely with more water and cooked 10 min at 126°C in an autoclave at 15 lb pressure. The cooked samples were then dried by air at 88°C for 24 hr. The raw and cooked material of each variety was ground in a Wiley mill to pass 40-mesh. The ground samples were kept under refrigeration at 4°C until needed for chemical determinations and biological assays.

AOAC (1950) methods were used in the chemical analyses. Thiamine content was determined by the thiochrome method of Hennessy and Cerecedo (1939), and riboflavin by the fluorometric method of Hodson and Norris (1939). The lysine, methionine, leucine, isoleucine, arginine, cystine, phenylalanine, and tyrosine contents were obtained by using Difco media and *Leuconostoc mesenteroides* P-60. Used for the cystine and valine analysis were synthetic media as recommended by Steel *et al.* (1949); and the threonine content was found with synthetic media and *Streptococcus faecalis* as microorganisms. Tryptophan was determined with *Lactobacillus arabinosus* and Difco media.

Biological trials. To determine the nutritive value of the eight varieties of cowpea, 54 weanling rats of the Wistar strain, from INCAP's animal colony, were divided into groups of 3 males and 3 females each. Their weight was distributed so that the average initial weight of the groups did not differ by more than 1 g. The rats were kept in all-wire cages with raised screen bottoms. Food intake and weight gain were recorded every seven days for 28 days. Food and water were given *ad libitum*. Table 1 shows partial composition of the experimental diets. The control was a soybean diet having the same protein level. All diets were supplemented with 4% of salt mixture, 5% of cottonseed oil, 1% cod liver oil, cornstarch tc make 100 g, and 4 ml of a vitamin solution (Manna and Hauge, 1953).

For the protein depletion-repletion experiment, adult rats, weighing 200 g, were fed a protein-free diet made of 86% cornstarch, 5% salt mixture, 5% cottonseed oil, 2% cod liver oil, and 4 ml of a complete vitamin solution (Manna and Hauge, 1953) until they lost 25% of their original weight. The animals were then distributed by weight into 9 groups of 3 males and 3 females each and fed experimental diets for 14 days. The diets were analyzed for nitrogen content in order to calculate their protein efficiency ratio (PER). A second rat experiment, using the procedure previously described, compared the growth-promoting value of five cowpea samples to that of cooked black beans fed at 10% protein level in the diet.

RESULTS

Table 2 shows the chemical composition of the cowpea samples before and after cooking. As can be seen, the cooked material showed 9.2% less nitrogen and 17.4% less crude fiber than the raw material, but there was no difference between the raw and cooked samples in the ether extract and

Table 1. Percentage composition of diets used in the biological trials.

Ingredients	Samples [a]								
	1	3	5	6	7	8	9	10	11
Cowpea	42.44	41.36	40.40	39.90	42.11	43.01	38.46	44.58	....
Soybean	....	....	....	....	....	....	....	....	20.00
Minerals [b]	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Cottonseed oil	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Cod liver oil [c]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Cornstarch	47.56	48.64	49.60	50.10	47.89	46.99	51.54	45.42	70.00
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Vitamin solution, [d] ml	4	4	4	4	4	4	4	4	4
% protein in diet	9.44	9.50	9.94	9.62	9.50	9.70	9.12	9.56	8.06

[a] No. 1 Calico Crowder, No. 3 Brown Eye Crowder, No. 5 Black Eye Peas, No. 6 Blue Goose, No. 7 Bunch Purple Hull, No. 8 Black Crowder, No. 9 Lady Peas, No. 10 Dixie Lee Peas, and No. 11 Soybean Flour (50% protein).
[b] Salt mixture, Hegsted, Nutritional Biochemical Corporation, Cleveland, Ohio.
[c] Mead Johnson, Evansville, Indiana.
[d] Manna and Hauge (1953).

Table 2. Nutrient content of eight varieties of cowpea.[a]

Substance	Raw			Cooked		
	Range	Av.	Std. dev.	Range	Av.	Std. dev.
Protein, g%	24.1–25.4	24.8	0.48	22.4–26.0	24.1	1.13
Ether extract, g%	1.1– 3.0	1.9	0.62	1.7– 2.1	1.9	0.14
Crude fiber, g%	5.0– 6.9	6.3	0.64	3.6– 6.3	5.2	0.87
Ash, g%	3.4– 3.9	3.6	0.17	3.2– 3.6	3.4	0.14
Thiamin, mg	0.41–0.99	0.74	0.22	0.22–0.39	0.28	0.05
Riboflavin, mg	0.29–0.76	0.42	0.14	0.12–0.36	0.20	0.10
Niacin, mg	2.51–3.23	2.81	0.26	1.40–1.69	1.55	0.10

[a] All values expressed on a 10% moisture basis.

ash content. The variation among the samples was not significant.

Table 2 also gives the thiamine, riboflavin, and niacin content of both groups of samples. It indicates that there were great variations in the vitamin content of both the raw and cooked samples. Furthermore, the cooking process caused a 62% loss of thiamine, a 52% loss of riboflavin, and a 45% loss of niacin.

Table 3 shows the essential amino acid content of the raw and cooked samples. With the possible exception of tryptophan, the variation for individual amino acids among all the samples was small. When individual amino acids in both raw and cooked cowpea samples were compared, only small differences were found.

The results in growth and PER from the first growth experiment, summarized in Table 4, indicated that the cowpea samples have different nutritive values. The weight gains varied from 53 to 26 g, and the PER from 2.30 to 1.42. The gain in weight was higher with the soybean diet than with any of the eight varieties of cowpea.

The second growth experiment, also presented in Table 4, showed that black beans (*Phaseolus vulgaris*) have a lower nutritive value than cowpea, as demonstrated by the lower growth and PER obtained with beans. In addition, the table shows the results obtained with adult rats, whose weight gains varied from 63 to 54 g in the 14-day experimental period. As in the previous experiments, soybean proved to be the best protein.

DISCUSSION

The chemical composition of the cowpea samples (*Vigna sinensis*) was found to be similar to that of beans (*Phaseolus vulgaris*), according to data presented by several workers in Latin America (Bressani *et al.*, 1954, 1961; Chavez *et al.*, 1952; Jaffé, 1950b; Tandon *et al.*, 1957). Other authors reported that riboflavin and niacin (De and Borai, 1949; De and Datta, 1951; Hoover, 1955; Jaffé, 1950b; Jenkins, 1954; Richardson *et al.*, 1950) showed the greatest variation among varieties.

The variation in essential amino acid content is smaller in cowpea than reported for beans (Bressani *et al.*, 1961). When the average of the essential amino acid content of the eight varieties of raw cowpea was compared with that of beans, it was found

Table 3. Essential amino acid content for eight varieties of cowpea.

Amino acids[a]	Raw (mg)			Cooked (mg)		
	Range	Av.	Std. dev.	Range	Av.	Std. dev.
Arginine	0.433–0.572	0.500	0.053	0.430–0.516	0.474	0.043
Histidine	0.169–0.236	0.213	0.019	0.176–0.256	0.217	0.026
Isoleucine	0.305–0.333	0.318	0.010	0.309–0.354	0.327	0.018
Leucine	0.434–0.543	0.484	0.031	0.427–0.541	0.497	0.036
Lysine	0.467–0.497	0.486	0.011	0.400–0.466	0.430	0.022
Methionine	0.074–0.082	0.079	0.010	0.074–0.091	0.079	0.006
Cystine	0.026–0.038	0.032	0.004	0.025–0.040	0.028	0.005
Phenylalanine	0.251–0.290	0.263	0.012	0.228–0.268	0.252	0.013
Threonine	0.242–0.281	0.251	0.013	0.228–0.255	0.239	0.009
Tyrosine	0.113–0.137	0.124	0.008	0.111–0.129	0.121	0.006
Tryptophan	0.058–0.082	0.068	0.010	0.058–0.084	0.068	0.010
Valine	0.252–0.368	0.314	0.033	0.251–0.375	0.302	0.018

[a] Amino acids expressed on g of amino acids/g of nitrogen.

Table 4. Results of biological tests with rats fed cooked cowpea protein.

Sample	Cowpea 1	2	3	4	5	6	7	8	Soybean flour
Growth experiment [a]									
Gain in weight	42	47	39	31	53	26	49	53	94
PER	1.79	2.18	1.90	1.54	2.29	1.42	2.30	2.16	3.55
Repletion experiment [b]									
Gain in weight	63	58	61	54	59	54	62	56	73
PER	2.44	2.35	2.51	2.20	2.38	2.15	2.64	2.23	3.11
Comparison between cowpea and black beans [c]									
Gain in weight	27	39	25	22	.....	.....	.....	33	13
PER	1.33	1.49	1.21	1.06	.....	.....	.....	1.47	0.71

[a] Average initial weight: 45 g.
[b] Average initial weight: 175 g.
[c] Average initial weight: 61 g.

that cowpea samples had more arginine, histidine, leucine, methionine, tyrosine, and valine, and less phenylalanine and threonine.

The cooking process affected the proximate chemical composition of the samples only slightly, but there was a significant loss in vitamin content. Similar results have been reported by several investigators, not only for beans but also for other leguminous seeds (Bressani *et al.*, 1954; Sherwood *et al.*, 1954).

Changes were small when the essential amino acid content of the raw cowpea was compared with that of the cooked samples. By comparing the essential amino acid composition of the cowpea samples with that of the FAO (1957) protein reference pattern, methionine was found to be the most limiting amino acid, while tryptophan and lysine were found in sufficient amounts. This is of practical importance if cowpea is to supplement corn protein efficiently, since it is well known that corn proteins are deficient in both lysine and tryptophan, and contain adequate amounts of methionine. Therefore, the proteins of the two staple foods should complement each other efficiently.

The results obtained with animals are interesting. Although the eight varieties showed only small differences in essential amino acid composition, the rat experiments indicated that there is a marked difference, probably due to variations in amino acid availability, among the eight samples tested. In general, the protein efficiency ratio was higher for the cowpea samples than for the beans. The highest protein efficiency ratio obtained with black beans by Bressani *et al.* (1963) was 1.20, whereas it was 0.71 in the present study. Richardson (1948) also found differences in nutritive value among cowpea samples he studied, while Chavez *et al.* (1952), using rats, reported a growth and PER equivalent to 82.9% of the nutritive value of pure casein.

Since cowpeas have a better nutritive value than common black beans and can be grown under wider environmental conditions, it would be desirable to learn whether they would have the same acceptance by undernourished populations in the Central American area if they were prepared like black and red beans. Previous studies (Bressani and Scrimshaw, 1961) have also indicated that cowpea protein complements corn protein efficiently, a factor of practical value for those areas where the human rural population is largely dependent upon corn protein for most of its protein needs.

REFERENCES

A.O.A.C. 1950. "Official Methods of Analysis." Assoc. of Official Agr. Chemists, 7th ed. Washington, D. C.

Borchers, R., and C. W. Ackerson. 1950. The nutritive value of legume seeds. X. Effect of autoclaving and the trypsin inhibitor test for 17 species. *J. Nutrition* **41**, 339.

Bressani, R., and N. S. Scrimshaw. 1961. The development of INCAP vegetable mixtures. I. Basic animal studies. *In*: Progress in Meet-

ing Protein Needs of Infants and Preschool Children. Washington, D. C., Natl. Acad. of Sci.–Natl. Research Counc. Publ. 843, p. 35.

Bressani, R., E. Marcucci, C. E. Robles, and N. S. Scrimshaw. 1954. Nutritive value of Central American beans. I. Variation in the nitrogen, tryptophan, and niacin content of ten Guatemalan black beans (Phaseolus vulgaris L.) and the retention of the niacin after cooking. *Food Research* **19**, 263.

Bressani, R., L. G. Elias, and D. A. Navarrete. 1961. Nutritive value of Central American beans. IV. The essential amino acid content of samples of black beans, red beans, rice beans, and cowpeas of Guatemala. *J. Food Sci.* **26**, 525.

Bressani, R., L. G. Elias, and A. T. Valiente. 1963. Effect of cooking and of amino acid supplementation on the nutritive value of black beans (Phaseolus vulgaris L.). *Brit. J. Nutrition* **17**, 69.

Busson, F., R. Carbiener, and B. Bergeret. 1959. Etude de la fraction protedique des graines de Vigna unguicula, Walp. *Qual. Plant Materiae Vegetabiles* **6**, 11.

Chavez, N., N. R. Teodosio, A. Gomez de Matos, Jr., C. A. Lima, and J. L. de Almuda. 1952. The proteins of the cowpea (Vigna sinensis) in nutrition. *Rev. brasil. med.* **9**, 603.

De, H. N., and S. C. Borai. 1949. Mechanism of biosynthesis of ascorbic acid during germination. *Indian J. Med. Research* **37**, 101.

De, H. N., and S. C. Datta. 1951. Biosynthesis of nicotinic acid during germination of cereals and pulses. *Indian J. Agr. Sci.*, **21**, 375.

F.A.O. 1957. Protein requirements. Report of the F.A.O. Committee. Rome, Italy, 24–31 October 1955. Food and Agr. Organization U.N. F.A.O. Nutritional Studies No. 16. Rome, Italy.

Finks, A. J., O. B. Jones, and C. O. Johns. 1922. The role of cystine in the dietary properties of the proteins of the cow-pea, Vigna sinensis, and of the field pea, Pisum sativum. *J. Biol. Chem.* **52**, 403.

Flores, M. 1961. Food patterns in Central America and Panama. *In*: Tradition Science and Practice in Dietetics. Proceedings of the 3rd International Congress of Dietetics. London 10–14 July 1961. Yorkshire, Great Britain, Wm. Byles and Sons Ltd. of Bradford. p. 23.

Hennessy, O. J., and L. R. Cerecedo. 1939. The determination of free and phosphorylated thiamin by a modified thiochrome assay. *J. Am. Chem. Soc.* **61**, 179.

Hodson, A. Z., and L. C. Norris. 1939. A fluorometric method for determining the riboflavin content of foodstuffs. *J. Biol. Chem.* **131**, 621.

Hoover, M. W. 1955. Influence of maturity and storage treatments upon the ascorbic acid content of the seeds of Southern peas. *Food Research* **20**, 469.

Jaffé, W. G. 1949. Limiting essential amino acids of some legume seeds. *Proc. Soc. Exptl. Biol. Med.* **71**, 398.

Jaffé, W. G. 1950a. Protein digestibility and trypsin inhibitor activity of legume seeds. *Proc. Soc. Exptl. Biol. Med.* **75**, 219.

Jaffé, W. G. 1950b. Variations in the vitamin content of seeds and their agricultural significance. *Rev. Sanidad y Asistencia Social* (Venezuela) **15**, 423.

Jenkins, W. F. 1954. Pash harvest changes in refrigerated and nonrefrigerated cowpeas. *Proc. Am. Soc. Hort. Sci.* **64**, 327.

Manna, L., and S. M. Hauge. 1953. A possible relationship of vitamin B_{12} to orotic acid. *J. Biol. Chem.* **202**, 91.

Orr, M. L., and B. K. Watt. 1957. Amino acid content of foods. Washington, D. C., U. S. Dept. Agr. Home Econ. Research Rept. No. 4.

Richardson, L. R. 1948. Southern peas and other legume seeds as a source of protein for the growth of rats. *J. Nutrition* **36**, 451.

Richardson, L. R., W. A. Brittingham, I. Whitacre, H. M. Reed, and E. Williams. 1950. Vitamin content of southern peas at different stages of maturity. *J. Am. Dietet. Assoc.* **26**, 34.

Sherwood, F. W., V. Weldon, and W. J. Peterson. 1954. Effect of cooking and of methionine supplementation on the growth-promoting property of cowpea (Vigna sinensis) protein. *J. Nutrition* **52**, 199.

Sohonie, K., and A. P. Bhandarker. 1954. Trypsin inhibitors in Indian foodstuffs. I. Inhibitors in vegetables. *J. Sci. Ind. Research* **13B**, 500.

Steel, B. F., H. E. Sauberlich, M. S. Reynolds, and C. A. Bauman. 1949. Media for Leuconostic mesenteroides P-60 and Leuconostoc citrovorum 8081. *J. Biol. Chem.* **177**, 533.

Tandon, O. B., R. Bressani, N. S. Scrimshaw, and F. LeBeau. 1957. Nutritive value of beans. Nutrients in Central American beans. *J. Agr. Food Chem.* **5**, 137.